Apical dominance and growth *in vitro* of *Alstroemeria*

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Content

Chapter 1

General Introduction

Micropropagation

The background

Plant tissue culture refers to the culture of plant cells, tissues or organs on a suitable artificial nutrient medium in a controlled environment under aseptic conditions. It includes techniques and methods used for botanical research and has several practical objectives. Haberlandt (1902) envisioned the concepts of plant tissue culture over a hundred years ago. With the first successes in developing plant tissue culture (Gautheret 1934, Nobecourt 1939, White 1934) and after the discovery of plant hormones, *viz.*, auxins in the 1930s and cytokinins in the 1950s, a new technology based on the manipulation of living plant material was ready in the 1950s. After that improvements have been relatively marginal. The initial aims were to use this technique as research tool. At the peak of the plant tissue culture era in the 1980s, many commercial laboratories were established around the world which mainly focused on micropropagation for mass production of clonal plants for the floricultural and horticultural industry. Applications of plant tissue culture techniques include micropropagation, various breeding techniques (*e.g.* haploids and genetic engineering), production of secondary metabolites and *ex-situ* conservation of valuable germplasms. Among these techniques, micropropagation is the most widely used.

The technology

Micropropagation is vegetative propagation using *in vitro* culture techniques. This technology was developed some fifty years ago. Micropropagation has rapidly been adopted in several countries all over the world and micropropagation protocols have been developed for a wide range of plant species. There are five stages in commercial micropropagation: 0 – preparation of mother plants, 1 – initiation of the culture, 2 – multiplication, 3 – elongation and rooting, 4 – transfer to the greenhouse (Debergh and Read 1991). Problems met during the successive stages and possible solutions are given in Table 1.

Table 1. The stages in micropropagation, their problems and possible solutions, partly based on Debergh and Read (1991) and George and Debergh (2008).

Stages	Possible solutions
0: preparation of mother plants	
• The plant material is internally populated by microorganisms.	- Mother plants are preferably grown under hygienic conditions such as in a greenhouse. - For tropical and subtropical crops, relatively high temperature (25ºC) and relatively low humidity (75%) are used. - Do not water by overhead irrigation.
• The plant material appears to be recalcitrant during initiation.	- Cold storage is needed for dormancy breaking of bulbous crops and trees. - Control photoperiod for the plants where flowering is under photoperiodic control. - Pretreatment with growth regulators (*e.g.*, cytokinins and gibberellins).
1: initiation of culture	
• The plant material responds poorly.	- Select suitable initial explants with the consideration of the age of stock plant, the physiological age of the explants and its developmental stage and size. - Adapt phase 0. - Limit the biosynthesis of phenolics and theirs oxidation by applying activated charcoal and antioxidants.
• The plant material is contaminated.	- Intensify surface-sterilization. - When the organ that is used for initiation is stress-resistant, a hot-water treatment may be applied. - Adapt stage 0. - NB antibotics stop the growth of microorganisms in the medium but usually do not remove internal micro-organisms.
2: multiplication	
• Poor multiplication	- Adapt the cytokinin treatment (type and concentration of cytokinin). - Use other plant growth regulators (auxin-transport inhibitors, stigolactone inhihibitors). - Use double layer.
• Hyperhydricity	- Decrease the RH in the headspace. - Increase the concentration of solidifier and/or change from Gelrite to agar. - Decrease the cytokinin concentration. - Adapt the inorganic formulation.
• Topnecrosis	- Increase Ca and/or increase evaporation from leaves.
• Senescence of shoots	- Use more aerated containers to minimize accumulation of ethylene in the headspace which causes plant senescence. - Capture ethylene from the headspace with $KMnO_4$.

Table 1. (cont.)

Stages	Possible solutions
3: rooting	- Reduce the costs in root induction by inducing the root ex vitro.
• Poor rooting	- Adapt the auxin treatment. - Adapt during stage 2 by elongation of the stems (double layer or etiolation).
• Shoot degeneration during rooting	- Capture ethylene from the headspace by $KMnO_4$.
4: transfer to greenhouse	
• Large losses during acclimatization and poor growth during and after acclimatization	- Gradually adjust in vitro shootlets to greenhouse conditions by controlling light and humidity and applying fertilizers and fungicides. - Pre-acclimatize plantlets during stage 3. - Adapt the rooting treatment.
• Off-types	- Avoid de novo formation of buds as the off-type plants may arise. - Avoid using excessive dosage of a cytokinin or inappropriate cytokinin which may cause epigenetic variations (e.g., hyperhydricity and bushiness). - Determine before the bulk number is produced whether multiplication system produces true-to-type plants but not off-type plants.
• Loss of chimerism	- Cannot use adventitious bud formation in stages 2 and 3 for chimera plants since segregation will occur. - Avoid using excessive dosage in stages 2 and 3 of a cytokinin or inappropriate cytokinin.

The methods to propagate plants *in vitro* are (1) axillary-bud branching, (2) adventitious shoot formation and (3) somatic embryogenesis (Brown and Thorpe 1995). The latter two produce large numbers of plantlets which have been regenerated adventitiously. Regeneration may be direct from cells in the explant or indirect via an intermediate callus phase. However, genetic and epigenetic variations may occur leading to off-type plants including dwarfs, color changes or mosaic patterns, growth habit changes, and changes in productivity (Thorpe and Harry 1997, Smulders and De Klerk 2011). Propagation via axillary-bud branching produces less plantlets because of the limited number of axillary buds and the limited number of axillary buds that actually grow out. Nevertheless, this method is the most widely used for commercial micropropagation, because it produces usually true-to-type plantlets, despite some exceptions. Epigenetic aberrations *e.g.*, bushiness has been developed in *Zanthedeschia* and gerbera, especially

when high cytokinin concentrations are used to induce axillary buds (Smulders and De Klerk 2011). In hosta, losing of chimeric charactistics has occurred in plants obtained from axillary shoot proliferation (Paek and Ma 1996).

Culture can be performed in solid and liquid culture systems. In the former, plants are cultivated on medium containing solidifying agents (usually agar or Gelrite). In the latter, plants are immersed in liquid medium that is usually agitated but may also be static. Recently, more productive liquid culture systems have been developed, making use of temporary immersion (Etienne and Berthouly 2002). So far, the solid culture system has been used for commercial micropropagation much more than the liquid culture systems despite the lower propagation rates. Even though the advantages of the liquid culture systems are evident *i.e.*, more rapid growth and propagation and the opportunity of scaling-up and automation, there are some disadvantages that limit their use: more complications to operate when using sophisticated bioreactors, occurrence of physiological disorders (hyperhydricity), high risk of microorganism contamination and often the increased maintenance costs and input of knowhow.

The potential success of micropropagation protocols depends -in addition to the genotype- on environmental factors (light and temperature) and medium factors (the composition with respect to nutrients and plant growth regulators). Although the nutrient compositions of the media have hardly changed for a long period, a better understanding of their consumption and rate of depletion by monitoring the nutrient medium has evolved. Many studies have been carried out to develop optimum medium compositions for specific plants. Most authors use MS formulation nowadays. Some authors though base the formulation on analysis of the compositions of plant tissues (*e.g.*, Nas and Read 2004, Jorge *et al*. 2007). With this approach, some crops grow and multiply better with the adapted medium (Bouman and Tiekska 2005). Currently, few relatively 'new' plant growth regulators are being employed successfully to improve growth and proliferation. These include thidiazuron which is in many cases more effective than other cytokinins, jasmonic acid and polyamines as morphogenic growth regulators, fluridone which helps in dormancy release of storage organs, and growth retardants which also inhibit ethylene and/or gibberellin biosynthesis and are able to enhance proliferation and control abnormality and hyperhydricity (Altman and Ziv 1997). Acclimatization is the most crucial stage for micropropagated plantlets. They have to survive conditions of higher light and lower

humidity than that experienced under *in vitro* conditions. The leaves of *in vitro* plantlets normally form less cuticular wax (Short *et al*. 1987), reduce vascular tissue development and have small palisade cell layers (Smith *et al*. 1986, Reuther 1988). The major defect that causes high water loss when tissue-cultured plants are exposed to dry air, is that stomata in vitrodo not close (Santamaria *et al*. 1993). Some approaches have been developed to solve these problems, for example, photoautotrophic systems (Kozai 1991) and the use of plant growth retardants which offers the production of more easily acclimatizable plantlets and plant structures produced in bioreactor systems (Ziv 1992).

In comparison to *ex vitro* vegetative propagation, the advantages of micropropagation are that per year several multiplication cycles can be carried out and that pathogens are absent during the propagation, so that the plants do not get infected. Beside the impressive successes of micropropagation, there are several limiting factors to its use (Wang and Charles 1991). The cost of the labor is high, up to 70% of the production costs of micropropagation. This makes micropropagation for many crops too expensive. The problems of hyperhydricity, acclimatization and contamination can cause great losses. Furthermore, genetic variation (such as polyploidy, aneuploidy and mutations) and epigenetic variation may result in the loss of desirable economic traits of tissue-cultured products. Finally, many genotypes are still recalcitrant.

Commercial micropropagation

The micropropagation industry started off during the 1970s and 1980s (Zimmerman and Jones 1991). In the history of commercial plant tissue culture, orchids were among the first species to be cultured (Hartman and Zimmerman 1999). Commercial applications of micropropagation in various species of horticultural plants are currently underway in many countries all over the world. Micropropagation protocols have been developed for a wide range of crops: ornamentals (*e.g.*, orchids, gerberas, roses, carnations and lilies), vegetables (*e.g.*, tomato, carrot and celery), food crops (*e.g.*, sugarcane, potato and cassava), fruits (*e.g.*, apple, strawberry, banana and pineapple), plantation crops (*e.g.*, coconut, cacao and tea) and spices (*e.g.*, cinnamon, ginger and turmeric).

Globally, the demand for clean healthy plant materials for agriculture, horticulture, forestry and ornamental industries is in excess of 16 trillion units per year, which equals US$ 4 trillion (Prakash 2009). For ornamentals, it is estimated that the global sale of cut flowers and pot plants is US$ 90 and 60 billion, respectively. The production of ornamentals by commercial micropropagation was in 1986 130 million plants globally. Currently, over one billion ornamentals are produced yearly through micropropagation (Prakash 2009). During the 1970s, ornamentals were mostly produced by commercial laboratories located in US and the Netherlands, but since the 1980s, production activity has migrated to Asia, Africa and Central America, due to lower production costs and improved transportation (Thorpe and Harry 1997). Global commercial micropropagation is still growing in recent years. Management and stock production, and research and development often stay in Europe (Winkelmann *et al*. 2006).

Statistical information on *in vitro* plant production worldwide is rare and not fully accurate. Detailed data collections can be found in the reports by Pierik (1987, 1988, 1991a and b, 1997), Pierik and Ruibing (1997) and O'Riordain (1999, 2002). Commercial tissue culture labs in Western Europe and North America were mainly established in the 1970s and 1980s (Winkelmann *et al.* 2006). In these countries, commercial micropropagation expanded in the 1980s (Zimmerman and Jones 1991, Holdgate and Zandvoort 1999). During the late 1980s, there were *ca.* 250 commercial micropropagation companies in Western Europe and also *ca.* 250 in US (Jones 1987, Pierik 1991c). However, in the early 1990s stagnation and even drastic declines were noticed. For example, the number of commercial laboratory in the Netherlands increased from 28 to 78 laboratories between 1983 and 1990 (Pierik, 1997). However, the number of laboratories declined to 67 between 1990 and 1995 (Pierik and Ruibing 1997). The reason was that many Dutch companies started production in low-wage countries, *e.g.*, India and China. The total number of tissue cultured plants used by the growers increased.

The major limitation of commercial micropropagation is high production costs. The cost of mass production varies with the species and depends on the method used. The costs for ornamentals are often not acceptable, whereas they are for some vegetables. The production costs for vegetables are 3-10 times that of the conventional methods (Thorpe 1990). The extension of micropropagation to species which are normally produced by seed propagation can be considered if production costs decrease. For future perspectives, it is

important that production costs are reduced to assure the future of micropropagation. This can be done by moving the production to zones where labor costs are low such as Asia, South America and Africa, or by developing new culture techniques which help to save labor costs such as temporary immersion systems as well as introducing automation and robotics (Sangwan-Norreel *et al*. 1991). Additionally, research on micropropagation protocols for woody plants, long-lived garden perennials and special novel ornamental hybrids seems to be commercially relevant, since in these plant groups the higher prices for tissue culture plants will be accepted by customers (Winkelmann *et al*. 2006).

Alstroemeria

Taxonomic description

Alstroemeria is known as Inca lily, Peruvian lily or lily of Lima (Stinson, 1952). It is a member of the family Alstroemeriaceae, order Liliales, superorder Liliiflorae, division Monocotyledonae (Dahlgren *et al*. 1985). The species in the genus *Alstroemeria* are mostly diploid with 16 chromosomes (2n=2x=16). The majority of *Alstroemeria* cultivars are triploids (2n=3x=24) with a few being either diploid or tetraploid (2n=4x=32) and aneuploidy occurring occasionally (Lakshmi 1980, Tsuchiya *et al*. 1987, Tsuchiya and Hang 1987, Hang and Tsuchiya 1988).

Alstroemeria is a native of South America mainly in Chile and Brazil and some species are found in Argentina, Paraguay, Bolivia, Peru, Ecuador and Venezuela (Ravenna 1988, Aker and Healy 1990). The center of distribution is in Chile (Bayer 1987). The habitats are wide, ranging of from the tropical Amazon area, the snowline of the Andes, high forests, desert sites, river valleys to coasts of the Pacific (Verboom 1979). Therefore, the genus tolerates many and often extreme environments.

Morphological description

The botanical features of the genus *Alstroemeria* are described by several researchers (Hutchinson 1959, Willis 1985, Bayer 1987). It consists of fleshy subterranean rhizomes, aerial shoots and roots. The leaves position alternately on either vegetative or generative stem and usually rotate 180° at the base, causing inversion of the lamina. The inflorescences are simple or compound cymes, each with one to five sympodially arranged flowers. The perianth is arranged into two whorls of three petals: inner and outer whorls. The petals of each whorl are different in size and shape. The ovary is pseudo-epigyn, trilocular and has axile placentation. The style is filiform and trilobed, becoming receptive after anther dehiscence. The ovules within each cavity of the ovary are numerous depending on the genotype. The mature fruit is a dry capsule that splits the round seeds explosively.

The roots of *Alstroemeria* vary from thin and fibrous to thick and tuberous, depending on the species (Buxbaum, 1951). The tuberous roots store starch and are edible (Mabberley 1987). The aerial shoots arise from the rhizomes that grow sympodially underground. Each aerial shoot has at its base two axillary buds. The first axillary bud of the aerial shoot always grows out. It grows out horizontally being the new rhizome apex and forms the next internode of the rhizome. At the next node, the rhizome apex changes the direction of growth, grows upwards and forms a new aerial shoot. The first axillary bud of this new aerial shoot develops as the previous one forming an internode of the rhizome. This process repeats itself. Thus a rhizome consists of a chain of basal internodes of successively developed shoots. The second axillary bud locates just above the first one. It may either stay dormant for a long time or develop into a lateral rhizome when the aerial shoot dies or is removed. No further axillary buds appear at the axils of higher leaves (Buxbaum 1951, Buitendijk 1998). Figure 1 explains the developmental stages of the rhizome and Figure 2 shows the morphology of *A. aurea* rhizomes.

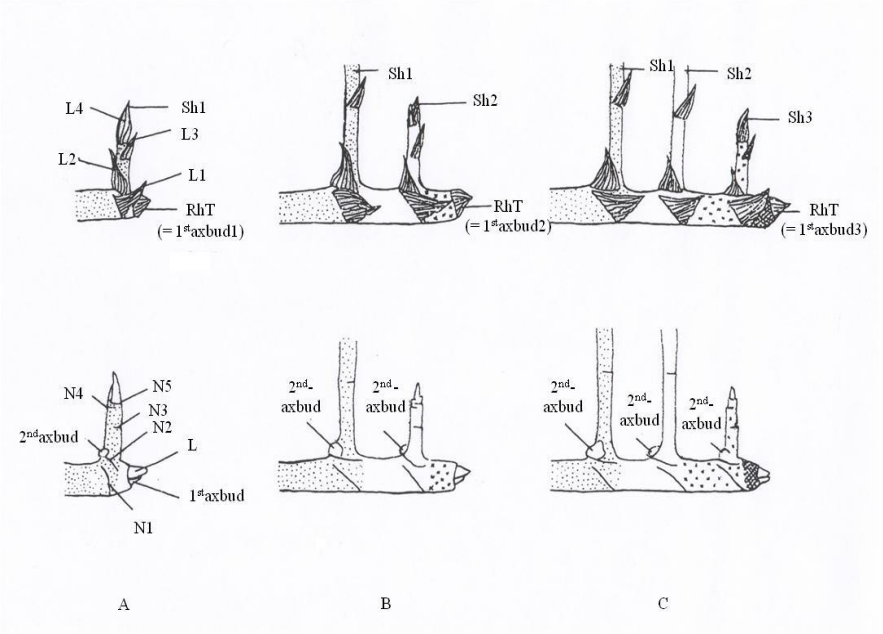

Figure 1. Three developmental stages of *Alstroemeria* rhizome (A through C), before (above) and after (below) removing the scale leaves at the nodes (modified after Buitendijk 1998). Axbud = axillary bud, L = leaf, N = node, RhT = rhizome tip, Sh = shoot. The number 1, 2, 3, 4, 5 indicate the subsequence of leaves and shoots.

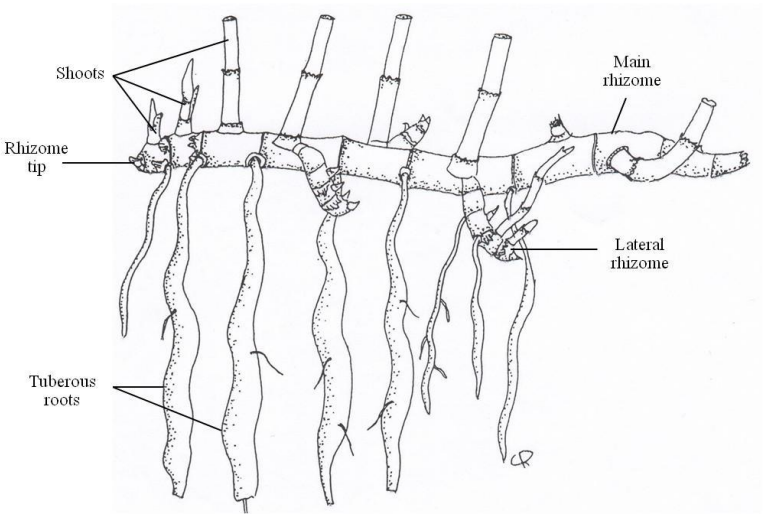

Figure 2. Morphology of the rhizomes of *A. aurea* (modified after Buitendijk 1998).

Economic importance

Several new hybrids of *Alstroemeria* have been introduced from Europe over the last four decades. An increased production of *Alstroemeria* cultivars worldwide has been noted, particularly in the Netherlands, US, UK and Japan. These plants have favorable characteristics such as high ornamental value, long vase life, variation in flower color and low energy input for cultivation (Healy and Lang, 1989, Healy and Wilkins, 1981). *Alstroemeria* cultivars have been released commercially as cut flowers in large numbers and in smaller numbers as pot and garden plants (Bridgen 1997). Currently, the world's two largest *Alstroemeria* producers are the Netherlands and Colombia. The total area of *Alstroemeria* production is estimated at 200-250 ha in Colombia and at 70 ha in the Netherlands (FloraCulture International 2009). Almost 95% of the products in Colombia are exported to the US. *Alstroemeria* is ranked at the 4th place of top selling in US's cut-flower market. In 2009, *ca.* 6% of the total number of produced flowers worldwide were imported to US costing 56 million US$ (Agriculture and Agri-Food Canada 2010). In the Netherlands, *Alstroemeria* is a long-time top occupant of most important cut flowers with the 8th and 11th places in 2003 and 2009, respectively (Table 2). The Dutch auction turnover in the year 2009 was 33 million Euros, which represented a decrease of *ca.* 23% compared to 2003 (Belgian Development Agency 2010). According to the data of the Dutch Flower Auction Association (VBN), the Netherlands sold *ca.* 104 million flower stems in European markets in 2009, which means a decline of *ca.* 10% compared to 2007 (Market News Service 2009).

Table 2. Main cut flowers on the Dutch auctions (Belgian Development Agency 2010).

2003	Turnover (€ million)	2009	Turnover (€ million)
1. *Rosa*	681	1. *Rosa*	696
2. *Chrysanthemum* (spray)	299	2. *Chrysanthemum* (spray)	233
3. *Tulipa*	186	3. *Tulipa*	199
4. *Lilium*	160	4. *Lilium*	141
5. *Gerbera*	106	5. *Gerbera*	107
6. Freesia	66	6. *Cymbidium*	60
7. *Anthurium*	60	7. *Chrysanthemum* (single)	58
8. *Alstroemeria*	43	8. *Freesia*	43
9. *Chrysanthemum* (single)	40	9. *Hippeastrum*	36
10. *Gypsophila*	38	10. *Eustoma*	35
11. *Dianthus*	37	11. *Alstroemeria*	33

In vitro propagation

Alstroemeria can be reproduced *in vivo* by seed and by rhizome division. Due to heterozygosity, homogeneous plant material cannot be obtained by seed reproduction (Pederson *et al*. 1996). Conventional propagation by rhizome division is a time consuming process, requires a large area of stock plants, and contributes to the spread of virus diseases (Van Zaayen 1995). After the introduction of micropropagation techniques, most *Astroemeria* propagation is now performed *in vitro* (Pederson *et al*. 1996).

A micropropagation technique based on rhizome branching has been developed to accelerate the multiplication efficiency (Ziv *et al*. 1973, Pierik *et al*. 1988, Van Zaayen *et al*. 1992, Bond and Alderson 1993). In this method, propagation is achieved by the outgrowth of the second axillary bud of the vertically growing shoot. However, the multiplication rate is still low at about 1.2–1.8 per cycle of 4 weeks. More recently, micropropagation methods as indirect somatic embryogenesis (Van Schaik *et al*. 1996, Lin *et al*. 2000, Akutsu and Sato 2002, Kim *et al*. 2006) and adventitious shoot regeneration (Lin *et al*. 1997, Lin *et al*. 1998, Pedraza-Santos *et al*. 2006) have been developed. However, despite its low multiplication rate the rhizome-branching method is more preferable and being used in most tissue culture companies to minimize variation of the propagated plants.

To increase the rhizome multiplication rate, several factors have been studied, for example, temperature, light, culture media, and growth regulators (Gabryszewska and Hempel 1985, Pierik *et al*. 1988, Bridgen and Winski 1989), but no significant improvements have been achieved. Only few researchers, *e.g.*, Bond and Alderson (1993) studied the effect of apical dominance on rhizome branching. They reported that decapitation of aerial shoot and rhizome tips significantly increased rhizome branching and suggested that the tips might have strong apical dominance over axillary buds thus preventing branching. The authors also used an auxin transport inhibitor, but the treatment did not produce differences in multiplication rate, suggesting that the mechanism of apical dominance in *Alstroemeria* is a complex matter (see also **Chapters 4 and 5**).

Scope of the thesis

In commercial micropropagation, *Alstroemeria* is propagated vegetatively via rhizome branching, which is achieved by forced outgrowth of the second axillary bud of the vertically growing shoot. Only this bud occasionally develops into a lateral rhizome. However, this preferred method gives a low multiplication rate due to strong apical dominance. This impedes the introduction of new genotypes. Up to now, very few studies have focused on apical dominance in *Alstroemeria* (*e.g*, Bond and Anderson 1993), even though it directly affects the outgrowth of axillary buds. These data imply that it is important to elucidate apical dominance mechanisms in *Alstroemeria*. This is to understand how it controls axillary bud outgrowth and how to improve multiplication factors in *Alstroemeria*.

Furthermore, it appears that the growth of *Alstroemeria* rhizomes *in vitro* is rather slow. This is also a major obstacle in *Alstroemeria* micropropagation. The size of lateral rhizomes should be large enough, so they can be separated from the main rhizome sooner. It is obvious that nutritional factors influence the growth of *in vitro* plantlets. In many cases, growing the plants with optimal medium constituents for specific species greatly enhances the growth (Badaoui *et al*. 1996, Kintzios *et al* 2007, Staikidou *et al*. 2006). Furthermore, exposure to moderate stress conditions results in better growth and multiplication (De Pereira-Netto and McCown 1999). According to observations in the greenhouse, the growth of storage organ rhizomes of *Alstroemeria* strongly increases after an abiotic stress. Therefore, a study on nutrient consumption and moderate abiotic stress may help to solve the slow-growth problem of *Alstroemeria* rhizomes.

Even though there are problems in other phases of *Alstroemeria* micropropagation, we only focus in this thesis on one particular stage: the multiplication phase (also known as stage 2). The general aim of this thesis was to improve micropropagation in *Alstroemeria* by enhancing rhizome growth and multiplication. The specific aim was to study basic and applied aspects of *Alstroemeria* rhizome micropropagation in the following main areas: (1) nutrient consumption during a micropropagation cycle as related to the growth of rhizomes; (2) moderate abiotic stress and its effect towards rhizome growth; (3) apical dominance mechanism(s) in the control of outgrowth of axillary buds. By performing research in these different areas useful clues

were obtained how to increase growth and multiplication rate of rhizomes in *Alstroemeria* micropropagation.

Nutritional factors obviously play an important role in rhizome growth. So far, only few studies have focused on nutrition of *Alstroemeria in vitro* (*viz.*, Elliott *et al*. 1993, De Klerk and ter Brugge 2010). In **Chapter 2**, a detailed study of rhizome growth and multiplication was made and this was linked to a determination of nutrient uptake during a subculture cycle. This provided information on elemental exhaustion. Furthermore, the elemental composition of rhizomes and shoots was analyzed. Finally, adaptation of the elemental composition of the nutrient medium according to the tissue analysis was investigated, aiming at enhanced rhizome growth.

Under moderate stress conditions, plants may initiate a response that helps them to resist a future severe stress (De Klerk and Pumisutapon 2008). Thus, they develop dormancy and form storage organs in order to survive an unfavorable season and to ascertain fast re-growth after that. This is a protection strategy of plants. In **Chapter 3**, plants were treated under moderate abiotic stresses: heat, cold, anaerobiosis, drought and salinity. This was to investigate whether *Alstroemeria* was able to enhance rhizome growth as well as multiplication after moderate stresses. Additionally, moderate stress was given to plants prior to severe stress.

The outgrowth of axillary buds is controlled by plant hormones. It has been long known that axillary bud outgrowth is inhibited by auxin which is synthesized by the young leaves at the apex and transported basipetally in the stem, and is promoted by cytokinins which are synthesized in the roots and transported acropetally in the stem. Recently, strigolactones (SLs) have been proposed as novel branching inhibiting hormones, which are believed to inhibit bud outgrowth via dampening of auxin transport. The SL mutants in *Arabidopsis* show an increased branching phenotype and have increased auxin transport capacity in the stem (Bennett *et al*. 2006, Ongaro and Leyser 2008). In **Chapter 4**, the effect of excision of shoot and rhizome tips was investigated. The reason for this was that the architecture of *Alstroemeria* is different from other plants because *Alstroemeria* has two main apices (the aerial shoot apex and the rhizome apex; the rhizome apex is actually the apex of an elongated lateral branch), and that the axillary bud that is inhibited is located at the aerial shoot and no at the rhizome. The investigations were done with auxin under the assumption that replacement of the apical tip by auxin-lanolin mixture would lead to

restoration of apical dominance. On the other hand application of auxin transport inhibitors was expected to promote axillary bud outgrowth. The role of other hormones including cytokinins and brassinosteroids was also examined. Subsequently, the role of the novel branching inhibitor SL was reported in **Chapter 5**. Inhibitors of SL biosynthesis such as fluridone and D2 were applied to the culture medium supposing that they would promote axillary bud outgrowth. In addition, inhibition by a synthetic SL, GR24, was either investigated alone or in combination with fluridone. The effect of auxin transport inhibitors on fluridone promotion of axillary branching was also examined since it has been claimed that inhibition of SL involves increased auxin transport. Furthermore, auxin transport levels in shoots and rhizome of the plants which received fluridone alone or received fluridone and GR24 were compared. Finally, results from all above chapters are discussed in **Chapter 6** and future perspectives on the improvement of *Alstroemeria* micropropagation are presented.

Chapter 2

Nutrient consumption in *Alstroemeria* cultured *in vitro*

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To be submitted

Abstract

Poor rhizome growth is a main obstacle in *Alstroemeria* micropropagation. We studied nutrient consumption during *in vitro* culture with the aim to improve rhizome growth. Growth and multiplication of the rhizomes, and nutrient consumption were compared in 10 and 25 ml solid MS media during 4 weeks. Both rhizome growth and multiplication were higher in 25 ml medium. The same quantity of sucrose was taken up in 10 ml and 25 ml but the quantity of inorganic nutrients taken up in 25 ml was twice as high as that in 10 ml. This can likely be explained by different diffusion rates of sucrose and inorganic nutrients and the thickness of the medium so that in 10 ml the diffusion-distances to the explant are larger. Moreover, the uptake rate by the explant may be larger for inorganics. NH_4^+ and $H_2PO_4^-$ were taken up most rapidly, and Mg^{2+} and Ca^{2+} were still at a high concentration after 4 weeks. This is due to the uptake rate by the explant. Elemental analysis of tissue-cultured plants indicated that Mg and P levels were relatively low, and Ca level was relatively high in rhizomes compared to shoots and stems. We hypothesized that decreasing Mg and P, and increasing Ca concentrations in the culture medium would improve rhizome growth. Only reducing Mg^{2+} to half standard level of MS formulation improved rhizome growth with *ca.* 30%. Rhizome growth was not better when $H_2PO_4^-$ concentration was decreased or Ca^{2+} concentration was increased compared to the control.

Keywords: nutrients, uptake, rhizome, culture medium, media adaptation

Abbreviations: BAP – 6-benzylaminopurine, MS – Murashige and Skoog formulation of inorganic nutrients, FW – fresh weight

Introduction

Alstroemeria is a high-value ornamental plant with a colorful appearance and an elegant shape. Hybrids are used for cut flower production and as potted plant (Bridgen 1997). Micropropagation is the preferred method for large-scale propagation and involves rhizome multiplication by forced outgrowth of axillary buds. With respect to the Dutch production numbers, *Alstroemeria* ranks at the 2nd position of micropropagated cut flowers in (Pierik and Ruibing 1997). The propagation factor though is low (less than two per cycle) due to strong apical dominance and slow growth of the rhizome. Nutritional factors obviously play a role in rhizome growth, but so far there have been only few studies on nutrition of *Alstroemeria in vitro* (*viz.*, Elliott *et al.* 1993, De Klerk and Ter Brugge 2010).

In plant tissue culture media, inorganic nutrients are main ingredients. Commonly, MS (Murashige and Skoog, 1962) is used. MS was originally developed for optimal growth of tobacco callus. Since MS seems usually satisfactory, most researchers apply this formulation as standard (George and De Klerk, 2008). Nevertheless, it is unlikely that MS is optimal for shoot growth in all kinds of other plant species. Dose-response studies to improve the formulation are very time-consuming because of the large number of components and because of interactions between components. There are other approaches to improve the elemental formulation, among others, identification of the elements that become exhausted and determination of elemental composition of plant tissues. Elemental consumption has been studied in various crops, often in cell cultures in liquid medium (Schmitz and Lörz, 1990; Holme, 1998) and occasionally in shoot cultures on semi-solid medium (Leifert *et al.* 1995; Ruzic *et al*., 2001). Elemental analysis of plant tissues may provide information on distinctive elemental compositions of plant species. This information can be used to adapt the nutrient formulation. Several authors have used formulations based on tissue analysis successfully (Badaoui *et al.* 1996, Kintzios *et al*. 2001, Gonçalves *et al*. 2005, Staikidou *et al*. 2006). Since different cell types accumulate certain elements in varying amounts (Conn and Gilliham, 2010), we hypothesize accordingly that adaptation of the formulation in conformity with differences in the elemental composition of shoots and rhizomes may promote their growth specifically.

In our previous studies on tissue culture of *Alstroemeria*, we examined axillary bud outgrowth and found that it is promoted among others by removal of rhizome and shoot

apices (Pumisutapon *et al*. 2011, **Chapter 4**), and by adding inhibitors of carotenoid biosynthesis (**Chapter 5**). We also observed that moderate abiotic stress treatments enhance rhizome growth and multiplication (**Chapter 3**). Here we report on nutritional factors. We research exhaustion of nutrients. It should be noted that such study has also a general relevance because detailed information about nutrient consumption by shoot cultures on semi-solid medium is still scarce. We also analyzed the elemental composition of rhizomes and shoots and adapted the elemental composition of the nutrient medium accordingly, aiming at enhanced rhizome growth.

Materials and methods

Plant material and culture conditions

Stock cultures of two *Alstroemeria* cultivars, '24098 2B' and 'Sara' were provided by Könst Alstroemeria (Nieuwveen, the Netherlands) and Royal Van Zanten (Rijsenhout, the Netherlands), respectively. To maintain the stocks, rhizomes were subcultured every four weeks. The larger part of the vertical-growing shoots was removed, and lateral rhizomes were separated from the main rhizomes when their size was large enough (>1 cm). The medium contained MS salts and vitamins (Murashige and Skoog 1962), 4% (w/v) sucrose, 9 µM 6-benzylaminopurine (BAP) and 0.2% (w/v) Gelrite (pH 5.8). The cultures were incubated at 19°C under cool white fluorescent light for 16 h photoperiod (30 µE m^{-2} s^{-1}).

In all experiments, we used explants consisting of a rhizome with two aerial shoots from which the larger part had been removed leaving *ca.* 1 cm stem. Five explants were cultured in a polystyrene jar with 25 ml solid medium, unless indicated otherwise.

Measurements of sucrose, total amount of inorganics, and individual elements

To solidified medium, the same volume of MiliQ water was added. After vigorous shaking, the gel was broken up into fragments (diameter < 2 mm) resulting in a suspension. The medium components were allowed to diffuse out of the fragments at room temperature for 48 h. During this period, the Gelrite fragments precipitated. Aliquots were

taken from the supernatant for analysis. This method showed almost 100% recovery of medium components.

The total amount of inorganic nutrients was estimated with an EC-meter (31 5i, WTW Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). MS medium contains 1.99 g l^{-1} mineral elements (N, K, P etc.) in addition to H, C and O and the EC values were expressed as the amount of mineral elements. The total amount of carbohydrates was measured with a digital refractometer (PAL-1; Agato, Tokyo, Japan). Details on the accuracy of both methods are in De Klerk and Ter Brugge (2011). Sucrose is likely hydrolysed to some extent into glucose and fructose but this hardly influences the refractometer measurements (De Klerk and Ter Brugge, 2011). Individual minerals were analyzed by the service laboratory of the Chemical Biological Laboratory for WUR–Soil Centre (Wageningen, the Netherlands) using SFA-$CaCl_2$ (NH_4^+ and NO_3^-) and ICP-AES (other minerals). For determination of inorganics in plant tissues, individual elements were analyzed by the same service laboratory as mentioned above, using destruction H_2SO_4/H_2O_2/Se and SFA-Nt/Pt (N and P), and destruction $HNO_3/HF/H_2O_2$ and ICP-AES (other elements).

Medium adaptation

The concentrations of selected macronutrients, *viz.*, Mg^{2+}, $H_2PO_4^-$ and Ca^{2+}, were adapted to 0, ¼, ½, 1 and 2 times the concentrations in MS medium. In MS, these nutrients are added as $MgSO_4$, KH_2PO_4 and $CaCl_2$. Thus adaptation of Mg^{2+}, $H_2PO_4^-$ and Ca^{2+} also changed the concentration of the cations or anions derived from the same salts. For example, $MgSO_4$ dissociates into Mg^{2+} and SO_4^{2-}. When the concentration of Mg^{2+} was adapted to ½ the concentration in MS (in MS: 1.5 mM) then SO_4^{2-} was also reduced to 0.75 mM. In this case, K_2SO_4 was used for compensation. Table 1 shows concentration of adapted Mg^{2+}, $H_2PO_4^-$ and Ca^{2+} and concentration of salts used for compensation.

Statistical analysis

For each determination, three polystyrene jars (ø 66 mm) containing the 10 or 25 ml medium with five explants each were used. Three jars were used per treatment. In total,

per treatment 15 explants were used. Data were recorded after 4 weeks (or when indicated weekly). In determination of rhizome growth (fresh weight increase), the weights of original and new lateral rhizomes were pooled. Dry weight was also determined and the results were the same as for fresh weight. The means were evaluated with Student *t*-test. In all figures, means of 15 determinations ± SEs are shown.

Table 1. Concentration of adapted inorganic macronutrients in MS media formulation and concentration of the salts used for compensation.

Macro-elements	Salt used in MS	Adapted concentration		Compensation salts
		x MS concentration	mM	
Mg[1]	$MgSO_4$	0	0	1.5 mM K_2SO_4
		¼	0.38	1.13 mM K_2SO_4
		½	0.75	
		1	1.5	-
		2	3	-
P[1,2]	KH_2PO_4	0	0	-
		¼	0.31	-
		½	0.63	-
		1	1.25	-
		2	2.5	-
Ca[1]	$CaCl_2$	0	0	5.98 mM KCl
		¼	0.75	4.49 mM KCl
		½	1.50	2.99 mM KCl
		1	2.99	-
		2	5.98	-

[1]There was no compensation for adaptation at the 2 x concentration.

[2] In adaptation of $H_2PO_4^-$ concentration, there was no compensation for K^+ instead of 20.05 mM K in MS.

Results

Growth and multiplication

Alstroemeria explants were grown on 10 and 25 ml MS-solid medium. Figure 1 shows growth and multiplication for cultivar '24098 2B'. There was already a marked difference in fresh weight (FW)-increase of rhizomes after one week ($P < 0.05$). After four weeks, the FW-increase of rhizomes was 70% higher in 25 ml medium than 10 ml medium ($P < 0.005$; Fig. 1A). In Figure 1B, the numbers of new shoots and rhizomes is shown.

Both were promoted by increasing the volume. After 4 weeks, the number of new shoots and rhizomes were respectively 40% ($P < 0.005$) and 25% (not significant; $P = 0.32$) higher in 25 ml medium than in 10 ml medium. The insert in Figure 1A shows that at 25 ml, the relative shoot weight (shoot FW as a percent of total plant FW) was consistently higher than at 10 ml. The cultivar 'Sara' showed the same trends (data not shown).

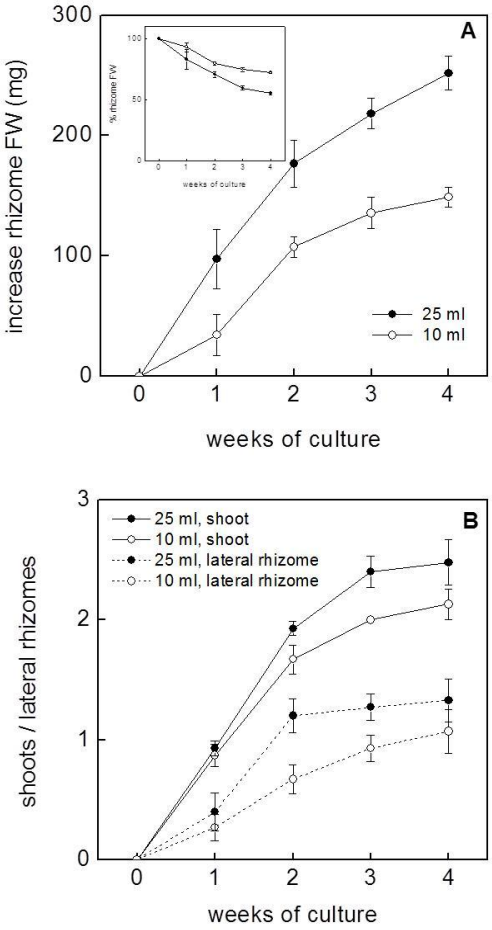

Figure 1. Rhizome growth (A) and shoot and rhizome multiplication (B) during 4 weeks of culture. Explants were grown on 10 and 25 ml solid MS medium. The insert in A shows the relative rhizome weight (rhizome FW as a percent of total plant FW)

Nutrient uptake

Figure 2 shows the uptake of total inorganic and organic nutrients as measured by an EC-meter and a brix meter, respectively. In 10 and 25 ml medium, 63% and 27% of the initial amount of sucrose -equaling 250 and 266 mg- were taken up during the 4 week-subculture, respectively. Thus, the quantity (mg) of sucrose taken up in both volumes was almost the same. With respect to inorganics, uptake was 68% and 58% -equaling 14 mg and 29 mg of mineral elements- for 10 and 25 ml, respectively. So, in 25 ml the quantity (mg) of inorganics taken up by the explants was twice as high as in 10 ml. For both inorganic and organic uptake, the major trends could be observed already after 1 week. The uptake by cultivar 'Sara' showed similar trends (data not shown).

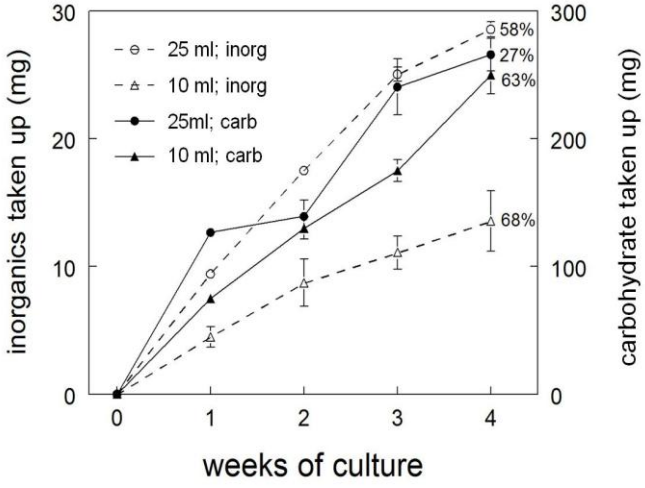

Figure 2. Uptake of inorganic nutrients and carbohydrates during 4 weeks of culture. Explants were grown on 10 and 25 ml solid MS medium. The uptake of inorganics was determined with an EC-meter and expressed as mg mineral elements (MS contains 1.99 g/l mineral elements). The carbohydrates were measured with a digital refractometer. Uptake after 4 weeks as a percentage of the total amount present at the start of culture is shown in brackets.

Individual inorganics were determined only after 4 weeks. Table 2 shows the ranking orders of nutrient uptake by cultivars '24098 2B'and 'Sara'. The orders of the various elements were similar in 10 and 25 ml medium and in both cultivars. For each element depletion in 10 ml medium was higher than in 25 ml medium, and depletion by '24098 2B' was slightly higher than by 'Sara'. Among the macronutrients, NH_4^+ and $H_2PO_4^-$ were taken up most rapidly. The macronutrient that still remained at a high concentration after 4 weeks was Ca^{2+}. For micronutrients, Fe^{2+} was taken up most rapidly, followed by Zn^{2+}, Mn^{2+} and Na^+, respectively. It should be noted that for Na^+, depletion was calculated relative to the concentration in MS but that the actual starting concentration was likely much higher because Gelrite itself contains a high level of Na^+ as contamination (George and De Klerk, 2008). The depletion of all inorganic nutrients taken together was very similar to the depletion measured with the EC-meter, showing the adequacy of the latter determination.

Table 2. Order of the uptake of inorganic nutrients by two *Alstroemeria* genotypes cultured on 10 and 25 ml MS solid medium after 4 weeks. In brackets is the percentage taken up from the medium after 4 weeks.

Medium volume	'24098 2B'	'Sara'
	Cationic macronutrient uptake	
10 ml	$NH_4^+ > K^+ > Mg^{2+} > Ca^{2+}$ (97%) (82%) (73%) (65%)	$NH_4^+ > K^+ > Mg^{2+} > Ca^{2+}$ (97%) (72%) (60%) (56%)
25 ml	$NH_4^+ > K^+ > Mg^{2+} > Ca^{2+}$ (82%) (61%) (48%) (34%)	$NH_4^+ > K^+ > Mg^{2+} > Ca^{2+}$ (84%) (54%) (41%) (35%)
	Anionic macronutrient uptake	
10 ml	$H_2PO_4^- > SO_4^{2-} > NO_3^-$ (97%) (91%) (88%)	$H_2PO_4^- > SO_4^{2-} > NO_3^-$ (96%) (86%) (83%)
25 ml	$H_2PO_4^- > SO_4^{2-} > NO_3^-$ (86%) (72%) (67%)	$H_2PO_4^- > SO_4^{2-} > NO_3^-$ (84%) (67%) (66%)
	Cationic micronutrient uptake	
10 ml	$Fe^{2+} > Zn^{2+} > Mn^{2+} > Na^{2+}$ (93%) (83%) (66%) (62%)	$Fe^{2+} > Zn^{2+} > Mn^{2+} > Na^{2+}$ (88%) (77%) (58%) (51%)
25 ml	$Fe^{2+} > Zn^{2+} > Mn^{2+} > Na^{2+}$ (89%) (61%) (39%) (34%)	$Fe^{2+} > Zn^{2+} > Mn^{2+} > Na^{2+}$ (88%) (58%) (38%) (36%)

Mineral composition of plant tissues

To compare the levels of nutrients in rhizomes and aerial parts (stems and leaves), the mineral compositions were determined in '24098 2B' *in vitro* grown plants. Table 3 shows the average levels of N, P, K, Ca, Mg and S. Just like in plants growing *ex vitro* (Epstein 1971), N was most abundant. In MS medium, N is also the most abundant mineral and the relative level in tissue-cultured plantlets is higher than in well-growing tissues ex vitro. The relative levels of the bivalent cations Ca^{2+} and Mg^{2+} were lower than in *ex-vitro* plants. It should also be noted that the level of the macroelements in tissue-culture plants (*ca.* 5 mol/kg DW) is considerably higher than the level in *ex-vitro* plants (1.5 mol/kg DW). There were obvious differences in the levels of Mg, P and Ca between rhizomes and aerial tissues. In rhizomes, Mg and P levels were relatively low, whereas Ca level was relatively high.

Table 3. Macroelemental composition of tissues of *Alstroemeria* '24098 2B' *in vitro* plants as compared to the composition of MS nutrient medium and well-growing plant shoots ex vitro. The composition is shown as mol/kg DW and in brackets as mol%.

Macro-elements	In *Alstroemeria* (mol/kg DW)			In MS (mol/l)	*In plant shoots *ex vitro* (mol/kg DW)
	Rhizome	Stem	Leaf		
N	4.46 (79.0 %)	3.38 (71.6 %)	3.83 (73.9 %)	0.06 (68.6 %)	1.0 (64.4 %)
P	0.06 (1.0 %)	0.09 (2.0 %)	0.12 (2.4 %)	0.00125 (1.4 %)	0.06 (3.9 %)
K	0.94 (16.7 %)	1.18 (23.9 %)	1.07 (20.5 %)	0.02 (23 %)	0.25 (16.1 %)
Ca	0.11 (1.9 %)	0.05 (1.0 %)	0.04 (0.8 %)	0.003 (3.4 %)	0.125 (8.0 %)
Mg	0.02 (0.4 %)	0.03 (0.6 %)	0.06 (1.3 %)	0.0015 (1.7 %)	0.08 (5.1 %)
S	0.06 (1.1 %)	0.04 (0.9 %)	0.07 (1.3 %)	0.0015 (1.9 %)	0.03 (1.9 %)
Total macro-elements	5.6	4.8	5.2	0.087	1.5

*Based on total nutrients (Epstein, 1971)

For the microelements, we analyzed only four, *viz.*, Fe, Zn, Mn and Cu (Table 4). All tissues contained Fe and Mn the most (except Mn level in leaves), followed by Zn and Cu, respectively. Fe and Cu levels were higher and Zn and Mn levels were lower in the normal, well-growing plant shoots (again except Mn level in leaves; calculation based on Epstein 1971) than *Alstroemeria*. There was not much difference in the level of each micronutrient between rhizome and aerial tissues. It should be noted that the level of the microelements in tissue culture (*ca.* 5 mmol/kg DW) was higher than the level in ex-vitro plants (3.4 mol/kg DW) but that the difference was less prominent than the difference in the macroelements. This may be related to the relative high level of microelements in MS (Tables 3 and 4).

Table 4. Microelemental composition of tissues of *Alstroemeria* '24098 2B' *in vitro* plants as compared to the composition of MS nutrient medium and well-growing plant shoots *ex vitro*. The composition is shown as mmol/kg DW and in brackets as mol% of the measured microelements.

Micro -elements	In *Alstroemeria* (mmol/kg DW)			In MS (mol/l)	*In plant shoots *ex vitro* (mmol/kg DW)
	Rhizome	Stem	Leaf		
Fe	3.12 (44.5 %)	1.39 (39.8 %)	3.08 (47.4 %)	0.1 (43.5 %)	2.0 (60.2 %)
Zn	0.92 (13.2 %)	0.66 (18.0 %)	1.70 (26.1 %)	0.03 (13.0 %)	0.3 (9.3 %)
Mn	2.86 (41.8 %)	1.52 (42.2 %)	1.62 (24.9 %)	0.1 (43.5 %)	1.0 (27.8 %)
Cu	0.04 (0.5 %)	0 (0 %)	0.11 (1.6 %)	0.0001 (0.4 %)	0.1 (2.8 %)
Total selected micro-elements	6.9	3.6	6.5	13	3.4

*From Epstein (1971)

Adaptation of macronutrients

Because tissue analysis showed marked differences in the levels of Mg, P and Ca amongst the rhizome and the aerial parts (Table 3), we adapted the nutrient medium accordingly. Since Mg and P were relatively low, and Ca was relatively high in the rhizome, we hypothesized that low Mg and P, and high Ca concentrations in the culture

medium might improve rhizome growth. Figure 3 shows the difference in rhizome growth when concentration of Mg^{2+} was adjusted. Reducing Mg^{2+} concentration to a half of the standard concentration (control) increased rhizome growth by *ca.* 30% (albeit not significant; $P = 0.31$). The lower concentrations (0 and ¼) decreased rhizome growth. Unlike Mg^{2+}, adaptation of P and Ca did not enhance rhizome growth according to the hypothesis. All P concentrations below the control decreased rhizome growth (data not shown). Rhizome growth was not improved when the Ca^{2+} concentration was increased to twice the normal concentration (data not shown). We also did similar examinations in cultivar 'Sara', but the effects were small (data not shown).

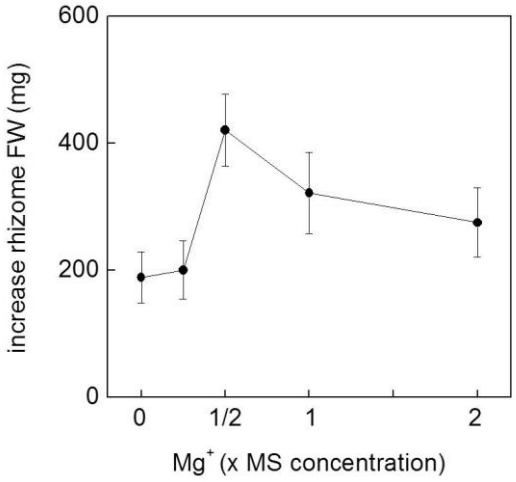

Figure 3. Rhizome growth after 4 weeks. Explants were grown on 25 ml solid MS medium with adapted Mg^{2+} concentration.

Discussion

Alstroemeria plantlets grown *in vitro* consist of a main rhizome, lateral rhizomes, shoots and often some roots. The rhizomes are used for subculturing since the shoots have no axillary meristems except for the one just above the rhizome. This axillary bud may grow out to form a lateral rhizome. Propagation is achieved by excision of lateral rhizomes that are sufficiently large. Thus, improvement of rhizome growth is necessary to enhance

Alstroemeria micropropagation. Moreover, in the final cycle large rhizomes should be produced to obtain robust propagules for transplanting to soil. Rhizome growth is promoted by the proper hormonal and environmental stimuli (*e.g.*, moderate stress, Pumisutapon *et al*. 2012, **Chapter 3**), and obviously by appropriate nutrition. Therefore we have examined nutrition in *Alstroemeria in vitro*. In addition, investigations of nutritional effects may be useful for the plants where apical dominance is strong. It has been postulated that limiting nutritional factors play a major role in regulation of some aspects of plant development and may provide an alternative to the concept of hormonal control (McIntyre, 2001).

Growth and nutrient consumption

When we grew *Alstroemeria* explants on different volumes of solid medium, both rhizome growth and multiplication were increased in 25 ml medium compared to 10 ml medium. Interestingly, increased growth in 25 ml was already apparent one week after subculture whereas at that time only 25% or less of the initial nutrient amounts had been taken up. The higher growth rate at 25 ml was probably caused by higher translocation of nutrients to the explants as compared to 10 ml medium. Solutes in the nutrient medium are translocated to the explants in two ways, *viz.*, by diffusion and by convection. The latter depends on water flow in the (semisolid) medium and will be low as transpiration from leaves in tissue culture is small (De Klerk 2010). The former, diffusion, is slow over large distances as described in Fick's law of diffusion (Taiz and Zeiger 2010). So, in the first week, uptake by diffusion will be limited to components that are at a distance of 1-2 cm from the explant. The amount of medium just below the cut end was smaller in 10 ml medium than in 25 ml medium because of medium thickness (2.9 *vs* 7.3 mm) so in 25 ml the amount of medium that can be used easily (because the distance to diffuse is small) is considerably larger. Uptake of medium components occurs mainly via the cut end because the epidermis is relatively impermeable (Leifert *et al*. 1995, Guan and De Klerk 2000).

The amount of solutes taken up by the explant depends on the translocation in the medium to the interface of explant and medium, but also on the uptake capacity of the explant, *e.g.*, the quantity of carriers. It is clear that for the inorganic nutrients, active uptake occurs. For example, uptake of NH_4^+ is consistently faster than uptake of K^+ (Table 2) whereas the Diffusion Coefficient (1.96) and the concentration (20 mM) of NH_4^+ and K^+

are the same. Inorganics were taken up by *Alstroemeria* faster than sucrose. This can be traced back to faster diffusion (for inorganics the mean Diffusion Coefficient is *ca.* 1.6 and for sucrose the Diffusion Coefficient is 0.52) and possibly also to different rates in active uptake. Uptake of sucrose in 10 and 25 ml medium was similar whereas uptake of inorganics was about twice as high in 25 ml. This indicates that the capacity of uptake by the explant was a limiting factor for sucrose as the available sucrose was 2.5 times higher in 25 ml.

In tissue-cultured *Alstroemeria*, the amount of minerals per 100 g DW is considerably higher in tissue-cultured plants than in *ex-vitro* cultured plants (5 mol/kg DW *vs* 1.5 mol/kg DW). This holds true for all crops analyzed thus far (G-J De Klerk, unp. results). It is not known whether this relative surplus of mineral elements influences the performances of tissue-cultured plants.

Determination of individual inorganics indicated that each element was taken up by *Alstroemeria* at different rate, and the order was similar to the order in liquid medium culture of *Alstroemeria* (De Klerk and Ter Brugge 2010) and the order in other crops (Leiffert *et al.* 1995). $H_2PO_4^-$ and NH_4^+ were used up most rapidly, while Mg^{2+} and Ca^{2+} were consumed slowly (Table 2). A very similar order of exhaustion is found in other cases where the tissues show fast growth (Schmitz and Lörz 1990). This suggests that the poor growth in *Alstroemeria* is unlikely caused by exhaustion of specific components.

Our observations also showed that a large portion of the nutrients was not yet taken up after 4 weeks (Fig. 2). Normally, the subculture cycle in *Alstroemeria* micropropagation is 4 weeks. This suggests that the subculture cycle may be extended, probably a few weeks.

Nutrient adaptation based on tissue analysis

In *Alstroemeria*, some inorganics occurred at a different level depending on plant parts. Remarkable differences between rhizomes and aerial tissues were low Mg and P and high Ca in rhizomes (Table 3). We hypothesized that when we lower Mg and P, and increase Ca, there might be increased rhizome growth. This hypothesis was formulated on the basis of the improved growth of various crops when the inorganic formulation in the nutrient medium was adapted according to the endogenous level in well-growing tissues

(*e.g.*, Bouman and Tiekstra 2005). However, the hypothesis was apparently incorrect with respect to improved growth of different plant parts. Adaptation of P and Ca in culture media did not bring about improved rhizome growth. This does not correspond to the finding by Elliott *et al*. (1993) that P at 1.25 (standard level in MS) and 2.5 mM increased rhizome growth relative to the lower concentrations. The hypothesis was somewhat correct for Mg, but the improvement was not statistically significant.

It should be noted that in crops with storage organs (bulbs: tulip and lily; H Bouman, Bulb Research Centre, Lisse, the Netherlands, pers. comm.), adaptation according to the endogenous elemental levels also did not improve growth. Plant storage organs are formed to conserve nutrients necessary for the growth of new shoots when the climatic conditions have become favorable. If some minerals would be stored in *Alstroemeria* rhizomes, they would be kept in special large molecules, *e.g.*, storage proteins and phytic acid, being "inert" and unnecessary for the proper functioning of the organ. So the hypothesis that the active level of some mineral should be high for proper functioning would be difficult to prove since a large part is in the inert, storage form. However, such storage concerns especially N, C and P. Other minerals are usually not accumulated in storage organs (Chapin *et al*, 1990). So this would only be a reason for the lack of an effect of P.

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Chapter 3

Moderate abiotic stresses increase rhizome-growth and propagation of *Alstroemeria in vitro*

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Abstract

Alstroemeria is multiplied *in vitro* by forced outgrowth of lateral rhizomes from rhizome explants. The multiplication rate is very low because of strong apical dominance and poor rhizome growth. We report here that moderate abiotic stresses stimulate both rhizome growth and outgrowth of lateral rhizomes and thus influence the multiplication rate positively. Heat stress was studied in more detail. Rhizome explants were exposed to heat in two ways, namely by a hot-water treatment (HWT) and by a hot-air treatment (HAT). Both increased rhizome growth when applied for 1 or 2 h in the range of 30 to 40ºC. The maximal enhancement was 75%. Because underground storage tissues like rhizomes are adaptations to survive climatic stresses, we presume that the increased sink-strength of rhizomes induced by moderate stress is related to stress adaptation. Moderate heat stress (38ºC HWT, 1 h) also resulted in protection of *Alstroemeria* plantlets from severe heat stress (45ºC HWT, 1-2 h) a few hours after the moderate stress. Several other abiotic stresses were also examined. Cold (0ºC) and anaerobiosis increased rhizome growth significantly whereas the increases with drought and salinity were not statistically significant. All abiotic stresses increased rhizome multiplication significantly and propagation maximally reached an almost double rate.

Keywords: rhizome, heat stress, cold stress, anaerobiosis, drought stress, salinity stress, hot-water treatment, hot-air treatment

Abbreviations: BAP - 6-benzylaminopurine, HAT - hot-air treatment, HWT - hot-water treatment, MS - Murashige and Skoog

Introduction

Alstroemeria hybrids are grown for cut flower and pot plant production (Bridgen 1997). Micropropagation is the preferred method for large-scale production of *Alstroemeria*. It involves the formation of lateral rhizomes brought about by stimulating outgrowth of axillary buds. Even though propagation *in vitro* is much higher than in the greenhouse, the propagation factor is still considered to be very low (usually far less than 2 per cycle). This is caused by strong apical dominance and poor rhizome growth.

According to *Alstroemeria* growers, rhizome growth strongly increases in greenhouse plants after an abiotic stress, *e.g.*, a change of temperature or fertilizer (R. Veenhof, Könst Alstroemeria, pers. comm.). Storage of resources in a "safe" organ to enable fast growth when the stress has ended, is a widespread adaptation to stress (Chapin III *et al.* 1990).

In a previous paper, we examined apical dominance in *Alstroemeria* cultured *in vitro* (Pumisutapon *et al.* 2011, **Chapter 4**). In this study, we show the positive effects of moderate heat stress and other moderate stresses on rhizome growth and multiplication of *Alstroemeria* cultured *in vitro*.

Materials and methods

Plant material and growth conditions

Cultures of the *Alstroemeria* hybrid '4962 2G' were obtained from Könst Alstroemeria (Nieuwveen, the Netherlands) and were used in most experiments. Two other cultivars, '24098 2B' (Könst Alstroemeria, Nieuwveen, The Netherlands) and 'Sara' (Van Zanten, Rijsenhout, The Netherlands), were used to examine applicability of the results in other *Alstroemeria* cultivars. The medium used for propagation of the stock and for all experiments comprised MS salts and vitamins (Murashige and Skoog 1962), 9 µM 6-benzylaminopurine (BAP), 4% sucrose and 0.2% (w/v) gelrite. The pH was adjusted to 5.8 before autoclaving (121ºC for 20 min). The cultures were grown at 19ºC and 16 h light per day (30 µE m^{-2} s^{-1}, Phillips TL 33). In all experiments, standard explants consisting of an intact rhizome with two decapitated shoots were used.

Heat treatments

For hot-water treatment (HWT), the explants were transferred to a container (polystyrene jar; ø 66 mm) with sterile water (5 explants/25 ml; water level *ca*. 7 mm) and incubated in a water bath (Lauda Bath Circulator C12). The HWT-temperatures were held within 0.3ºC. For hot-air treatment (HAT), the explants were placed on standard medium (5 explants/25 ml) and the jars with explants were placed in an incubator (Labcon LTIM 10). The HAT-temperatures were held within 1.0ºC. The explants were cultivated on standard medium after the heat treatments.

The beneficial influence of pretreatments with moderate warm-water stress was investigated using the procedure for *Arabidopsis* seedlings (De Klerk and Pumisutapon 2008). Explants were pretreated at 38ºC for 0 and 1 h. After that they were kept on standard medium at room temperature for 4 h before giving severe heat stress at 45ºC for 0, 1, 2 and 3 h.

Other stresses

Other abiotic stresses included cold, anaerobiosis, drought and salinity. For cold stress, the explants were submerged in sterile water (5 explants/25 ml) and incubated in the dark at 0ºC in a foam box containing ice for 0, 4, 8 or 24 h. For anaerobiosis treatment, the explants were submerged in sterile water (5 explants/25 ml) and kept in the dark at 19ºC for 0, 8, 24, 72 or 216 h. Drought stress was given by keeping the explants on dry filter paper in the laminar air flow cabinet for 0, 0.5, 1 or 2 h. Salinity stress was carried out by submerging the explants in a NaCl solution (500 mM; 5 explants/25 ml) for 0, 1, 2 or 4 h and rinsing with sterile water before transfer to standard medium.

Statistical analysis

In each determination, three jars (ø 66 mm) containing 25 ml medium with five explants each were used. Rhizome growth (fresh weight increase) and multiplication (the number of new lateral rhizomes; see Pumisutapon *et al*. 2011) were determined after four weeks. After prolonged/severe stress treatments some explants died. In these treatments, the

means refer to the surviving explants only. In the figures, means of 15 determinations ± SEs are shown. For rhizome growth, dry weight increase was also determined and the results were the same as for fresh weight. The means were evaluated with the Student *t*-test.

Results

Effect of abiotic stresses on rhizome growth

All explants survived the HWT when treated for 1 or 2 h at temperatures up to 42.5ºC. However, after 2h at 42.5ºC rhizome growth decreased indicating persistent damage (Fig. 1A). Temperatures between 36 and 39ºC increased rhizome growth maximally by *ca*. 50% compared with the control. The 2 h HWT gave a better result than the 1 h HWT (Fig. 1A).

Since HWT explants were submerged, they may have suffered from anaerobiosis. To examine this, the explants were submerged in increasing water volumes during the HWT. Figure 1B shows that the lowest water volume, 5 ml (water level *ca*. 1.5 mm), in which the explants were only partially submerged, resulted in the highest increase of rhizome growth. In 25 ml water (water level *ca*. 7 mm), the explants were just submerged. Increasing the water volume to 100 ml totally submerged the explants (water level *ca*. 30 mm) and the rhizomes did grow only little (Figure 1B). Moreover, many explants died (data not shown).

For HAT, explants were incubated at a range of temperatures for 1 h either without or with solid medium in the jars. Figure 2 shows that HAT between 30 and 40ºC increased rhizome growths. When the plants were treated without medium, rhizome growth increased less. Maximum growth promotion (75%) was reached for the 1 h treatment at 35ºC with solid medium. Increasing temperature up to 45 and 50ºC reduced rhizome growth (Fig. 2) and many explants died (data not shown).

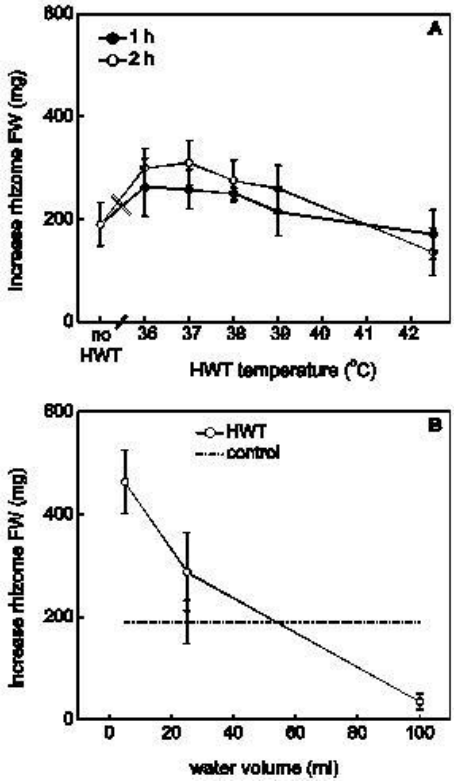

Figure 1. Rhizome growth after an HWT of rhizome explants at increasing temperatures for 1 and 2 h in 25 ml water (A) or at 38ºC for 1 h in different water volumes (B; control was no HWT at 25 ml water). After the HWT, the explants were grown on standard medium for 4 weeks.

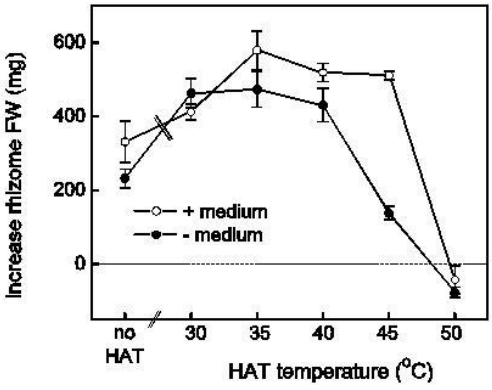

Figure 2. Rhizome growth after an HAT of rhizome explants at different temperatures for 1 h. HAT was given to explants in jars without or with medium. After the HWT, the explants were grown on standard medium for 4 weeks.

To compare HWT and HAT in more detail, explants were treated at 38ºC for 1-3 h. The HWT gave slightly better results (Fig. 3). To check whether the increase by moderate heat stress was limited to the cultivar used in the present examinations ('4962 2G'), two other cultivars, '24098 2B' and 'Sara', were also given HWT and HAT. They showed similar results (data not shown).

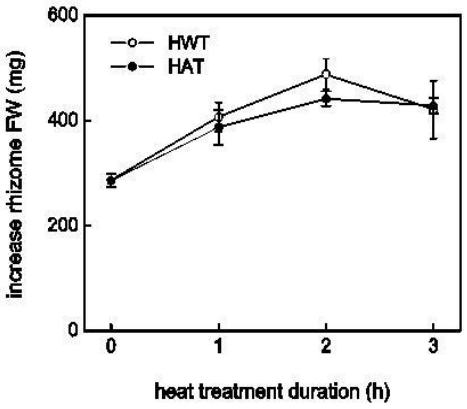

Figure 3. Rhizome growth after HWT or HAT of rhizome explants at 38ºC for 0-3 h. After the HWT/HAT, the explants were grown on standard medium for 4 weeks.

We also examined other moderate abiotic stresses, namely cold (0ºC), anaerobiosis, drought and salinity. All resulted in increased rhizome growth. Under the stress conditions used, cold stress gave the highest promotion of growth (97%; $P < 0.0005$). Anaerobiosis promoted rhizome growth by maximally 38% ($P < 0.005$), drought stress by 35% (not significant; $P = 0.30$), and salinity stress by 14% (not significant; $P = 0.39$). Protracted stress treatments resulted in less growth promotion and often in death. In Figure 4, the data with cold and anaerobiosis are shown.

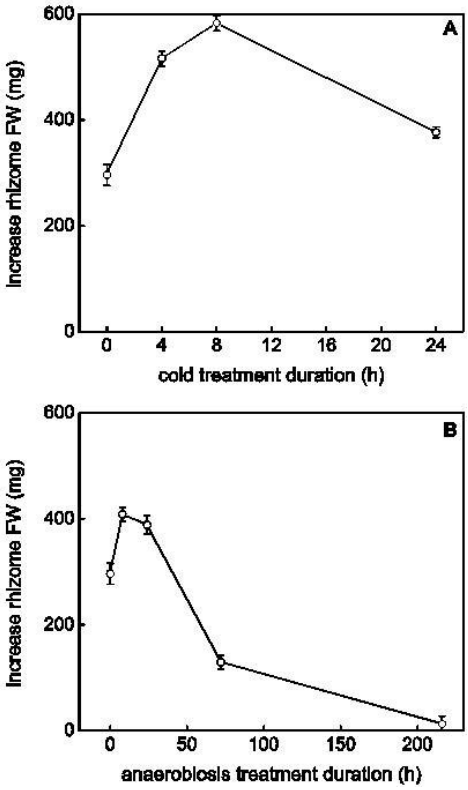

Figure 4. Rhizome growth after cold stress (0ºC) for 0-24 h (A) or anaerobiosis stress (19ºC) for 0-216 h (B). After the stress, the explants were grown on standard medium for 4 weeks.

Moderate HWT protects against severe heat stress

Plants that grow under stressful conditions often allocate a high proportion of vegetative biomass to "safe" storage organs (Chapin III *et al*. 1990). At the same time, moderate pre-stress is a well-known procedure to protect plants from a subsequent severe stress (De Klerk and Pumisutapon 2008). We therefore tested whether a 1 h HWT (that increases the growth of the storage organ, Fig. 1A) also offers rhizome explants protection from severe stress. So the explants were given a 1 h HWT at 38ºC and 4 h later a severe HWT at 45ºC for 1, 2 or 3 h. Figure 5 shows plant survival after one week. Almost all rhizomes that received the HWT at 38ºC survived the 1 h HWT at 45ºC, whereas almost all

control rhizomes (no 38ºC HWT) died ($P < 0.0001$). After 2 h HWT at 45ºC, the control rhizomes died for 100%, whereas the 40% ($P < 0.005$) of the rhizomes pretreated with a 38ºC HWT survived. Only after 3 h at 45ºC, also 100% of the pretreated rhizomes died.

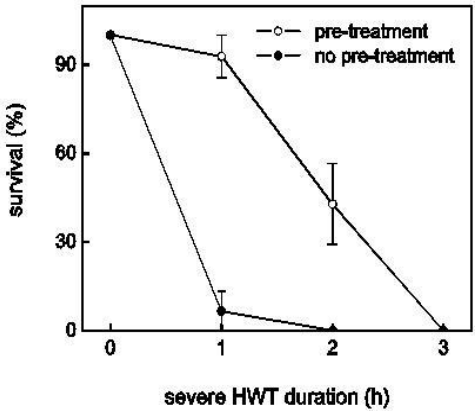

Figure 5. Survival of explants after an HWT at 45ºC for 1-3 h (severe stress) without or with a preceding HWT at 38ºC for 1 h (moderate stress). The moderate HWT was given 4 h before the severe HWT. After the HWT, the explants were grown on standard medium for 4 weeks.

Effect of abiotic stresses on rhizome propagation

Besides enhancing rhizome growth, abiotic stresses also increased the outgrowth of lateral rhizomes, even though the promotive effect was in most cases smaller. The optimal conditions for the different stresses were a 1 h HWT at 38ºC (50% increase), a 1 h HAT at 35ºC (52% increase), a 0.5 h drought treatment (23% increase), an 8 h cold treatment (23% increase), and a 1 h salinity treatment (67% increase) (for all $P < 0.05$). Only anaerobiosis did not cause a significant increase. As an example, the effect of HWT is shown (Fig. 6). It should be noted that whereas the effect of stresses on rhizome growth was relatively stable in consecutive experiments, the effect on lateral rhizome outgrowth was more variable.

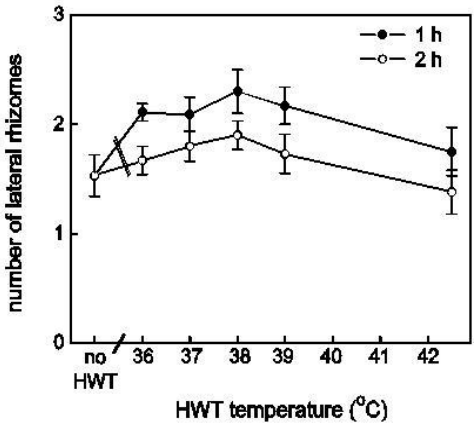

Figure 6. Rhizome propagation in explants treated with an HWT at increasing temperatures for 1 or 2 h. After the HWT, the explants were grown on standard medium containing 9 µM BAP for 4 we

Discussion

Improvement of rhizome growth

When plants are exposed to a moderate stress, they may initiate a response that renders them resistant to a future severe stress. This has been observed for, *e.g.*, moderate drought (Villar-Salvador *et al*. 2004) and heat stress (De Klerk and Pumisutapon 2008) and occurs both *in vitro* and *in vivo.* Protection is instantly but short-lived. Plants have also protection strategies for protracted adverse periods. Thus, they develop dormancy and form storage organs to survive an unfavorable season and to ascertain fast regrowth after that. Many plants that grow in stressful habitats, have increased mass allocation to the storage organs (Chapin III *et al*. 1990; Ryser and Urbas 2000; Hutchings and John 2004; Puijalon *et al.* 2005; Puijalon *et al*. 2008). *Alstroemeria* responds to moderate heat stress similarly by increasing rhizome growth. We have also measured shoot growth after the moderate stress, and there was no increase but actually often a decrease (data not shown). So the moderate stress does not lead to a general increase of growth but specifically to an increase of the sink activity of the rhizome. To the best of our knowledge, there are no other reports on the enhancement by a moderate stress of storage organ development such as tubers, bulbs, corms or rhizomes. Severe stress reduces the growth of storage organs. Heat stress

lowers potato tuber yields through reduced partitioning to the tubers and through reduced photosynthesis (Ewings 1981). Growth of onion (*Allium cepa* L.) bulbs is reduced by salinity stress with increasing NaCl concentration up to 100 mM (Chang and Randle 2004).

The mechanism by which the moderate stress enhances sink activity of rhizomes is not known. Signaling molecules which have been reported to regulate plant responses to heat stress include abscisic acid, salicylic acid, ethylene and hydrogen peroxide (Larkindale and Huang 2005), calcium ions (Knight 2000), and jasmonic acid (Clarke *et al*. 2009). Among these signaling molecules, abscisic acid (Kim *et al*. 1994), ethylene and jasmonic acid (Jásik and De Klerk 2006; Rayirath *et al*. 2011) play an important role in plant storage organ formation.

Hot-water and hot-air stress

We applied heat stress through HWT and HAT. For HWT, the explants were transferred to a small jar with water and the jar was placed in a water bath. For HAT, the tissue culture container was transferred into an incubator. Both efficiently improved growth and multiplication of *Alstroemeria* rhizomes (Figs. 1, 2 and 6) and HWT gave a slightly better effect (Fig. 3). Possibly better heat conduction of water and additional moderate stress by anaerobiosis during HWT are involved (Jackson 1985; Dolferus *et al*. 2003). In this respect it should also be noted that it is difficult to control the temperature within an incubator and that an incubator should be used with sufficient air circulation. Accurate temperature control is highly important, as the effect of temperature may depend on a few degrees ºC (in *Arabidopsis* seedlings, a rise of 1ºC for 2 h leads to an increase of the death rate from 10-20% to almost 100%; R. Stolker and G.J. De Klerk, unp. results).

When the tissues are submerged during the HWT, they also suffer from severe anaerobiosis because the high temperature increases the metabolic rate (so the production of CO_2 and the requirement for O_2). The solubility of gases decreases with temperature (by *ca.* 1/3 when the temperature increases from 20 to 40ºC). The importance of adequate uptake and emission of gases by submerged plant tissue during HWT, is demonstrated by the sharp decrease of survival of *Arabidopsis* seedlings when the number of seedlings per jar increases (De Klerk and Pumisutapon 2008). In *Alstroemeria*, increasing the water volume resulted in a reduction of rhizome growth (Fig. 1b) and in increased incidence of

death (data not shown). Explants were totally submerged in the highest water volume (100 ml), but only partially in the lowest water volume (5 ml).

For HAT, the increase of rhizome growth was less (Fig. 2) and the incidence of death was higher when explants were heated in the empty jars, compared to those that were heated in jars containing solid medium. Possibly, solid medium is necessary in HAT to prevent the tissues from dehydration. Furthermore, HAT increased rhizome growth slightly less than HWT when explants were treated at the same temperature level and durations (Fig. 3). This suggests that anaerobiosis during HWT is involved and causes an additional effect.

Enhancement of lateral rhizome outgrowth

All abiotic stresses also increased rhizome multiplication. In bulbous plants, abiotic stress such as heat increases the formation of bulbs and roots of chives (Fölster and Krug 1977). A short period of heat stress (32/25ºC day/night for five consecutive days) given to greenhouse grown potatoes decreases apical dominance and results in the development of many branches (Hammer *et al*. 1989). In pineapple, HWT at 60ºC for 6 or 8 min to dormant axillary buds before transfer to nutrient medium increases percentage bud-responsiveness and shoot emergence (Broomes and McEwan 1994). In the tropical fruit tree *Hancornia speciosa*, the multiplication rate of shoot culture is low because of strong apical dominance that cannot be overcome by cytokinin treatment. However, an increase in culture temperature from 31 to 35ºC over a 4-week period suppresses elongation and induces branching of all shoots, and a two-week period at 35ºC followed by culture at 31ºC leads to vigorous branching (De Pereira-Netto and McCown 1999). In red raspberry (*Rubus idaeus* L.), endodormancy in the canes growing in pots is released after HWT at 45ºC for 1 h (Rantanen and Palonen 2010). A heat treatment is also used to break dormancy in seeds (Farooq *et al*. 2008). These observations indicate that heat treatment induces bud outgrowth and can overcome apical dominance. Abiotic stresses may cause an increase in endogenous cytokinin level in plants accompanied by down-regulation of the activity of the main cytokinin degrading enzyme cytokinin oxidase/dehydrogenase (Rivero *et al*. 2009; Dobra *et al*. 2010). Abiotic stresses may also reduce the activity of the apical bud thereby reducing its ability to inhibit outgrowth of the axillary bud. The outgrowth of daughter bulbs in tulip

after a heat treatment is an extreme example as in this case the apical bud often deceases (Rees, 1971).

Conclusion

Moderate stress is a usable method to enhance the growth of *Alstroemeria* rhizomes *in vitro* and an increase of 70% may be obtained. The increase of growth is likely based on a protective adaptation by the rhizomes. The outgrowth of axillary buds into lateral rhizomes is promoted as well by moderate stress. Before commercial use, though, possible after-effects of the treatments should be examined first.

Acknowledgements

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Chapter 4

Hormonal control of the outgrowth of axillary buds in *Alstroemeria* cultured *in vitro*

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Abstract

We study apical dominance in *Alstroemeria*, a plant with an architecture very different from the model species used in research on apical dominance. The standard explant was a rhizome with a tip and two vertically growing shoots from which the larger part had been excised leaving *ca.* 1 cm stem. The axillary buds that resumed growth were located at this 1-cm stem just above the rhizome. They were released by removal of the rhizome tip and the shoot tips. Replacement of excised tips by lanolin with indole-3-butyric acid (IBA) restored apical dominance. The auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and *N*-1-napthylphthalamic acid (NPA) reduced apical dominance. 6-Benzylaminopurine (BAP) enhanced axillary bud outgrowth but the highest concentrations (> 9 µM) caused fasciation. Thidiazuron (TDZ) did not show improvement relative to BAP. Even though the architecture of *Alstroemeria* and the model species are very different, their hormonal mechanisms in apical dominance are for the greater part very similar.

Keywords: apical dominance, auxin, auxin transport inhibitors, cytokinin, rhizome, sympodial growth.

Abbreviations: 2^{nd}axbud - second axillary bud; BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; NPA - *N*-1-napthyl-phthalamic acid; RhT - rhizome tip; Sh1 and Sh2 - shoot most far from and close to the RhT, respectively; ShT1 and ShT2 - shoot tip of Sh1 and Sh2, respectively; TDZ - thidiazuron; TIBA - 2,3,5-triiodobenzoic acid.

Introduction

Alstroemeria is a rhizomatous monocotyledonous species. Hybrids are grown for cut flower production, as bedding plants and as pot plants (Bridgen 1997). The growth habit of *Alstroemeria* is sympodial. At each node, the apex of the rhizome changes direction of growth from horizontal to vertical, generates an aerial shoot and stops growing. The axillary bud located at the node grows into a rhizome. Thus, the rhizome is actually a chain of first internodes of side shoots. The second, next higher axillary bud at the vertical shoot (2^{nd}axbud) usually remains dormant because of strong apical dominance but occasionally grows into a lateral rhizome (Van Schaik 1998). In spite of a low multiplication rate (1.2 - 1.8 per 4 weeks), forced outgrowth of the 2^{nd}axbud is the preferred method for micropropagation of *Alstroemeria* (Pedersen *et al.* 1996, Buitendijk *et al.* 1992).

Apical dominance is the control exerted by the shoot apex over the outgrowth of axillary buds. Auxin produced in the shoot apex is transported basipetally and indirectly inhibits axillary bud outgrowth. Cytokinin promotes the outgrowth of axillary buds. Recently, research on mutants in *Arabidopsis*, pea and petunia presented evidence for a novel branching-inhibiting, carotenoid-derived growth regulator (Ongaro and Leyser 2008). This inhibitor has been identified in pea (Gomez-Roldan *et al.* 2008) and rice (Umehara *et al*. 2008) as strigolactone. In addition, other phytohormones have been reported to influence outgrowth of axillary buds, such as ethylene (Haver and Schuch 2001), gibberellic acid (Tamas *et al.* 1989), abscisic acid (Cline and Oh 2006) and brassinosteroids (Pereira-Netto *et al.* 2006).

From the theoretical point of view, the regulation of apical dominance in *Alstroemeria* is intriguing because of the plant's architecture. *Alstroemeria* plants have two types of major apical buds, *viz.*, shoot- and rhizome-tips. With respect to the position at the stem, the shoot tip is located above the inhibited axillary bud, and the rhizome tip below. Bond and Alderson (1993) have reported that both shoot and rhizome tips control the outgrowth of the 2^{nd}axbud, but that the auxin transport inhibitor TIBA has no effect. They conclude that the mechanism of apical dominance in *Alstroemeria* is complicated. We have initiated a research into the mechanism of apical dominance and rhizome growth in *Alstroemeria*. In this article, we examine the role of shoot tips, rhizome tip, auxin, cytokinins, and brassinosteroids.

Materials and methods

Cultures of the *Alstroemeria* L. cvs. '24098 2B' (used for cut flowers) and 'Sara' (used as pot plant) were obtained from Könst Alstroemeria (Nieuwveen, The Netherlands) and Royal Van *Zanten* (Rijsenhout, The Netherlands), respectively. Their genetic background is different, but their performance in tissue culture is alike. All experiments were carried out with both cultivars. The results were very similar and therefore we only show the results with cv. '24098 2B'.

The medium was Murashige and Skoog (1962; MS) salts and vitamins, 9 µM benzylaminopurine (BAP), 4 % sucrose and 0.2 % (w/v) Gelrite. The pH was adjusted to 5.8 before autoclaving (121°C for 20 min). The cultures were kept at 19°C and a 16-h photoperiod (irradiance of 30 µmol m^{-2} s^{-1}, TL 33 Philips, Eindhoven, The Netherlands). The standard explant was a rhizome consisting of two nodes with two aerial shoots from which the larger part had been excised leaving *ca.* 1 cm stem (see Fig. 1B). After 4 weeks of culture, the larger part of the shoots was cut off. Lateral rhizomes were separated from the main rhizome when sufficiently large (> *ca.* 1 cm). Several types of explants were used: an intact rhizome with two intact shoots (+RhT,+2ShTs; Fig. 1A) and an intact rhizome with two decapitated shoots (+RhT,-2ShTs; the standard explant in commercial propagation; Fig. 1B). The two aerial shoots were designated as Sh1 (the one most far from the rhizome tip) and Sh2 (the one close to the rhizome tip).

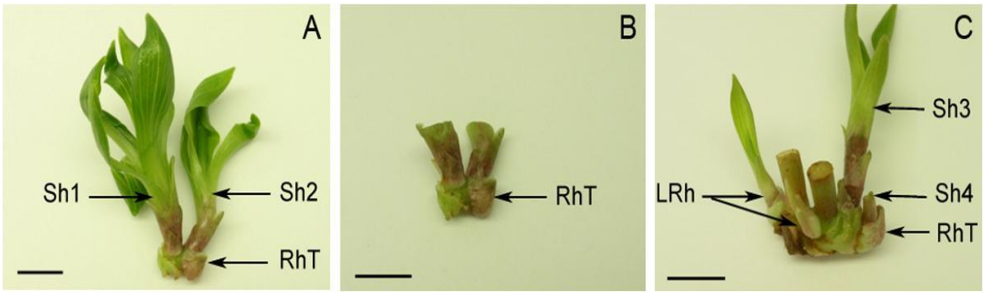

Figure 1. *Alstroemeria* explants used in this study: +RhT,+2ShTs (A) and +RhT,-2ShTs (B). Sh1 and Sh2 are the shoots located far from and close to the rhizome tip (RhT), respectively. When the explants were grown on standard medium for four weeks (C), new shoots (Sh3 and Sh4) might be formed, and the 2ndaxbuds at Sh1 and Sh2 might resume growth and develop into lateral rhizomes (LRh). All bars are 10 mm.

Auxin transport inhibitors (2,3,5-triiodobenzoic acid, TIBA, and *N*-1-napthylphthalamic acid NPA), cytokinins (BAP and thidiazuron, TDZ), and brassinosteroids (ergosterol and 24-epicastasterone) were added at the indicated concentrations. All growth regulators except BAP were added after autoclaving. Indole-3-butyric acid (IBA) was added as IBA-lanolin paste to the cut ends after excision of the shoot and/or the rhizome tip. The paste was prepared by dissolving IBA powder in 95 % ethanol, mixing with lanolin on a hot plate, followed by autoclaving.

For each determination, five polystyrene jars (66 mm) containing 25 cm^3 medium with three explants each were used. Data were recorded after four weeks. The effects were evaluated with χ^2-test and in some experiments by determination of the significance of the slope of the regression line. All experiments were repeated at least once.

Results

Effect of cytokinins and of the excision of shoot and rhizome tips

Cytokinins are successfully used for micropropagation *via* axillary bud proliferation in many plant species (*e,g.*, Gatti 2008, Sanatombi and Sharma 2008). In *Alstroemeria*, the axillary bud located at the aerial shoot just above the rhizome (2^{nd}axbud) may resume growth (Fig. 1C). With increasing BAP concentration, first the 2^{nd}axbuds at Sh1 and after that the ones at Sh2 resumed growth. At Sh1, almost all 2^{nd}axbuds were released, but at Sh2 less than 50 %, also at the highest BAP concentration (Fig. 2). 18 µM BAP caused high fasciation in the next subculture (data not shown). In the following experiments, 9 µM BAP was always added. In many crops, TDZ gives better results in multiplication than BAP (*e.g.*, Khurana-Kaul *et al*. 2010). In *Alstroemeria*, TDZ did not increase outgrowth but outgrowth was even somewhat reduced (data not shown).

To investigate the role of both types of apical buds, we excised the aerial shoot tips and/or the rhizome tip. Figure 3 shows that on standard medium with 9 µM BAP, apical dominance of the 2^{nd}axbud in Sh1 was broken in all explant types. The percentage of 2^{nd}axbud outgrowth at Sh2 was low (20 %) in intact explants (+RhT,+2ShTs) and increased after decapitation of both shoot and rhizome tips to 80 % (Fig. 3). Removal of the rhizome

tip resulted in more outgrowth than removal of the aerial shoots ($P < 0.05$). Excision of ShT2 increased outgrowth of the 2ndaxbud at Sh2 but not at Sh1 (data not shown).

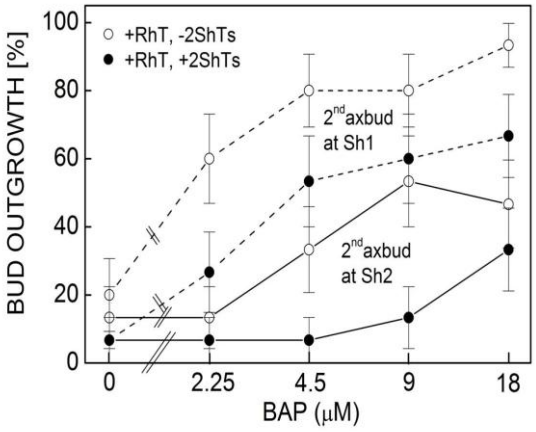

Figure 2. Outgrowth of 2ndaxbuds in different types of explants, +RhT,-2ShTs and +RhT,+2ShTs at Sh1 (dashed line) or Sh2 (solid line). Explants were grown for 4 weeks in standard medium containing 0, 2.25, 4.5, 9 and 18 µM BAP.

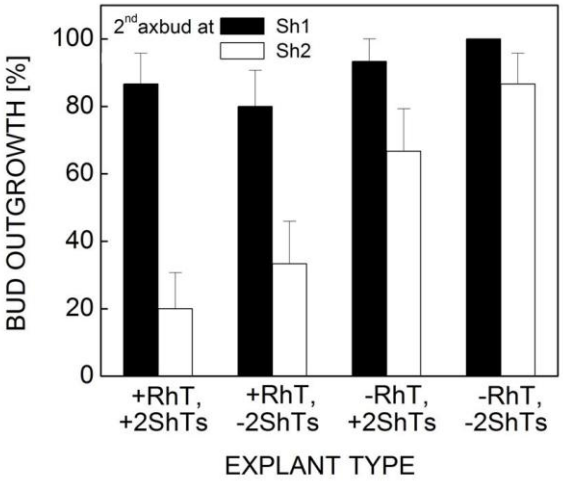

Figure 3. Outgrowth of 2ndaxbuds at Sh1 and Sh2 of different types of explant. Explants were grown on standard medium containing 9 µM BAP for 4 weeks.

Role of auxin

The apical tip is the primary source of auxin. In the classical experiments on apical dominance, lanoline paste with auxin restored apical dominance after decapitation (Thimann and Skoog 1933) and this method is still being used today (Cline 2000). We applied lanolin paste with increasing concentrations of IBA to the cut ends of shoot- and rhizome-tips in explants from which both types of tips had been cut off (Fig. 4A). The 2ndaxbud of Sh2 was almost completely inhibited at 10 mg g^{-1} IBA and the 2ndaxbud of Sh1 at 100 mg g^{-1} IBA. When lanolin paste with 30 mg g^{-1} IBA was applied to the cut ends of either shoot tips, rhizome tip or both, IBA reinstated apical dominance in all cases (Fig. 4B). Again, outgrowth of the 2ndaxbud at Sh2 was most strongly inhibited. When IBA-lanolin was added to only one of the shoot-cut ends of the two cultivars the 2ndaxbud located at the same shoot was inhibited but the effect on the other 2ndaxbud was minor (Fig. 5).

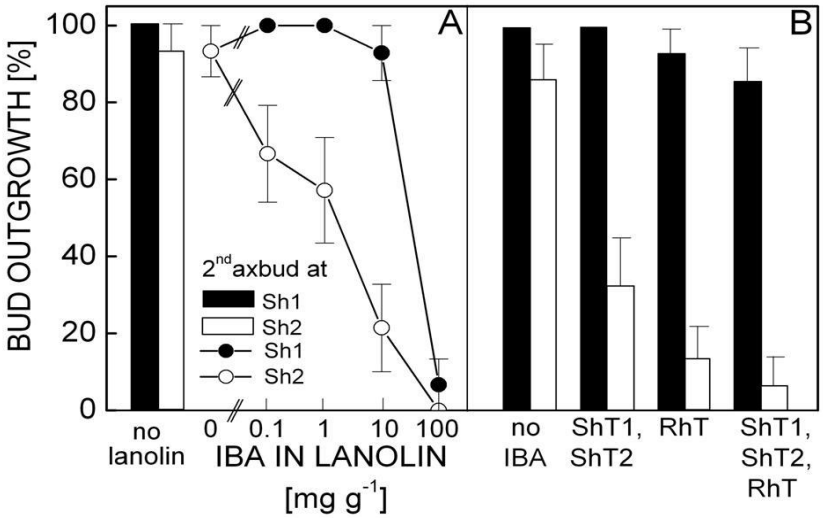

Figure 4. Outgrowth of 2ndaxbuds at Sh1 and Sh2 of explants from which both shoot tips and rhizome tips had been excised (-RhT,-2ShTs). At the cut ends, lanolin with increasing concentrations of IBA was applied (A). In B, 30 mg g^{-1} IBA-lanolin was applied to cut ends at indicated sites and at the other sites lanolin without IBA. Explants were grown in standard medium

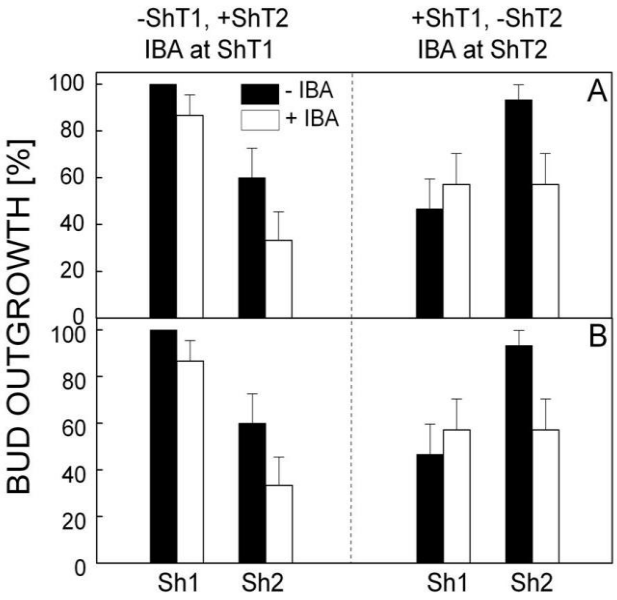

Figure 5. Outgrowth of 2[nd]axbuds at Sh1 and Sh2 of different explant types in cvs. '24098 2B' (A) and 'Sara' (B). In all explants, the rhizome tip was removed, and either the tip of Sh1 or the tip of Sh2 was excised. 30 mg g^{-1} IBA + lanolin or lanolin without IBA was applied as indicated. Explants were grown in the standard medium containing 9 µM BAP for 4 weekscontaining 9 µM BAP for 4 weeks.

Release from apical dominance by auxin transport inhibitors has been reported frequently (Nakajima *et al.* 2001). Therefore, TIBA was added at increasing concentrations to +RhT,+2ShTs and +RhT,-2ShTs explants. An increase of the TIBA concentration enhanced outgrowth of the 2[nd]axbud at Sh2 in both explant types (Fig. 6; slope of the regression line is significantly different from zero, $P < 0.05$). In Sh1, outgrowth was already close to 100 %, and the achieved promotion was not statistically significant (slope of the regression line is not significantly different from zero, $P = 0.14$). Similar results were obtained with NPA, another auxin transport inhibitor (data not shown).

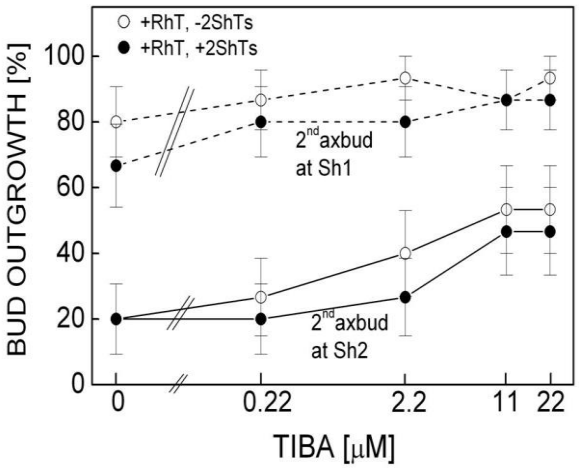

Figure 6. Outgrowth of 2[nd]axbuds in two type of explants, +RhT,-2ShTs and +RhT,+2ShTs at Sh1 (dashed line) or Sh2 (solid line). Explants were grown for 4 weeks on medium containing 9 μM BAP and increasing concentrations of TIBA.

Effect of other regulators

Other plant growth regulators have been implicated in apical dominance. Brassinosteroids are promising candidates (Pereira-Netto *et al.* 2006). Therefore, we have tested ergosterol and 24-epicastasterone. Both enhanced outgrowth, but the effects were small and statistically significant (data not shown).

Discussion

In contrast to the model plants in research on apical dominance (pea, *Arabidopsis* and petunia) that have one primary shoot apex, *Alstroemeria* has two types of main apical buds: aerial-shoot tips and rhizome tips. On basis of developmental origin, the shoot tip of *Alstroemeria* is a genuine apical bud and the rhizome tip originates from outgrowth of an axillary bud. Decapitation of both tips resulted in promotion of axillary bud outgrowth, removal of the rhizome tip having a larger effect than removal of the aerial-shoot tip. Excision of both had an additive effect (Fig. 3). Thus both tips control outgrowth, albeit

with different intensity. Excision of the apical bud at Sh2 or Sh1 only had little or no positive effect on the outgrowth of the 2ndaxbud at Sh1 or Sh2, respectively (data not shown). It should be noted that the axillary bud that is activated (in this paper denoted as 2ndaxbud) is located at the aerial shoot just above the rhizome. In other species, lower axillary branches may also inhibit outgrowth of upper ones and *vice versa* illustrating the bi-directional action of the inhibitory signals (*e.g.*, Ongaro *et al.* 2008). In the rhizomatous grass *Elytrigia repens*, axillary bud outgrowth is also significantly increased by excision of both parent shoot and rhizome tip (McIntyre and Cessna 1998).

Inhibition of outgrowth is restored by replacing the excised apical bud with lanolin containing auxin (Thimann and Skoog 1933). In *Alstroemeria*, IBA-lanolin restored apical dominance after excision of both the shoot and rhizome tips (Fig. 4A and 4B). Furthermore, auxin transport inhibitors (TIBA, Fig. 6 and NPA, data not shown) increased 2ndaxbud outgrowth. This observation does not correspond with the finding of Bond and Alderson (1993) that TIBA had no effect. However, these authors pooled the data of outgrowth at all shoots whereas we observed a significant effect at Sh2 only. When the data of both shoots are pooled, this effect becomes undetectable by the massive outgrowth at Sh1 (which is close to 100 %). Both TIBA and NPA have been reported to promote the axillary bud outgrowth in other species (Nakajima *et al.* 2001). Together, these data show both shoot tip and rhizome tip act *via* basipetally transported auxin.

The outgrowth of the 2ndaxbud at Sh1 was always higher than that at Sh2 (close to rhizome tip). Similar differences have been found in other species. Morris *et al.* (2005) observed that pea buds at the lower nodes grow out rapidly after decapitation of the shoot tip. This may be related to the IAA level that is lower in the basal nodes (Morris *et al.* 2005). Bud dormancy may also play a role. In *Phalaenopsis*, excised young buds from the highest position at the floral stalk have a reduced capacity to grow *in vitro*, so a higher rate of dormancy (Tanaka and Sakanishi 1978). Since in *Alstroemeria* Sh1 is older than Sh2, the 2ndaxbud at Sh1 is older and less dormant than the one at Sh2.

We also examined the roles of various growth regulators. It is obvious that auxin plays a role in the inhibition of axillary bud outgrowth in *Alstroemeria*. Auxin unlikely inhibits directly, but acts *via* upward-moving second messengers, *viz.*, cytokinin that promotes and a carotenoid derived compound that inhibits the axillary bud (McSteen and Leyser 2005). Both BAP and TDZ promoted outgrowth of 2ndaxbud, but not to 100 %. This

suggests that the level of apical dominance in *Alstroemeria* is strong. Other recently proposed candidates for axillary bud stimulation include brassinosteroids (Pereira-Netto *et al*. 2006). In *Alstroemeria,* both ergosterol and 24-epicastasterone increased 2ndaxbud outgrowth but only a little.

The two cultivars examined in our study gave very similar results in spite of their different genetic background. Thus the observed hormonal mechanisms in apical dominance probably apply to many other *Alstroemeria* genotypes. Future studies will include the role of strigolactone.

Acknowledgements

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Chapter 5

The role of strigolactone in apical dominance in *Alstroemeria* cultured *in vitro*

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To be submitted

Abstract

We studied the role of strigolactone (SL) in the regulation of axillary bud outgrowth in *Alstroemeria* cultured *in vitro*. Rhizomes with two intact aerial shoots and rhizomes from which the aerial shoots had been largely removed were used as explants. There is only one category of axillary buds that is blocked by apical dominance and that may resume growth. These axillary buds are located at the basal part of the aerial shoots just above the rhizome. Fluridone and D2 (both inhibitors of SL biosynthesis) increased axillary bud outgrowth. The SL analogue GR24 restored apical dominance. The auxin transport inhibitors *N*-1-napthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) promoted axillary bud outgrowth in the absence of fluridone but were inhibitory in its presence. This indicates that SL acts via inhibition of auxin transport. Indeed, when SL biosynthesis in the explants was inhibited by adding fluridone, basipetal auxin transport increased strongly (350-400%). Auxin transport was reduced when GR24 was applied together with fluridone.

Keywords: apical dominance, auxin transport, fluridone, axillary bud outgrowth, strigolactone

Abbreviations: 2ndaxbud - second axillary bud, CCD - carotenoid cleavage dioxygenase, IAA - indole-3-acetic acid, NPA - *N*-1-napthyl-phthalamic acid, RhT - rhizome tip, PATS - polar auxin transport stream, Sh1 and Sh2 – aerial shoot most far from and close to the RhT, respectively, Sh3 - newly formed shoot after Sh2, ShT1 and ShT2 - shoot tip of Sh1 and Sh2, respectively, SL - strigolactone

Introduction

Apical dominance refers to the control exerted by the primary shoot apex over the outgrowth of axillary buds. It has been known for decades that auxin and cytokinin play major roles in apical dominance inhibiting and promoting the outgrowth of axillary buds, respectively. Recently, a novel branching-inhibiting hormone has been discovered and identified as strigolactone (SL) (Gomez-Roldan *et al.* 2008, Umehara *et al.* 2008). The biosynthesis of SLs involves the following orthologous genes: *MORE AXILLARY GROWTH* (*MAX*) in *Arabidopsis*, *RAMOSUS* (*RMS*) in pea and *DECREASED APICAL DOMINANCE* (*DAD*) in petunia. The *max1, max3, max4, rms1*, *rms5 dad1* and *dad3* mutants all have an increased branching phenotype (see for recent review Domagalska and Leyser 2011).

In *Arabidopsis max* mutants, the auxin transport capacity is increased because PIN auxin efflux carriers are up-regulated (Bennett *et al.* 2006, Crawford *et al.* 2010). It was hypothesized that because of this axillary buds can export auxin into the main stem and that this enables them to resume growth (Domagalska and Leyser 2011). Alternatively, it has been proposed that SL is a second messenger: auxin promotes the expression of SL biosynthetic genes, SL enters the axillary bud and inhibits outgrowth (Brewer *et al.* 2009). Since SLs are derived from carotenoids, the effect of endogenous SLs will be reduced by inhibitors of carotenoid biosynthesis, for example, fluridone and norflurazon (Matusova *et al.* 2005). Inhibitors of later steps in the SL biosynthetic pathway also affect SL production, for example, inhibitors of carotenoid cleavage dioxygenase (CCD)7 and CCD8. These inhibitors are hydroxamic acid-type and some of them (*i.e.*, D2, D4, D5 and D6) increase branching in *Arabidopsis* (Sergeant *et al.* 2009).

Alstroemeria is a rhizomatous ornamental plant. Hybrids are grown for cut flower and potted plant production (Bridgen 1997). The axillary bud located at the aerial shoot just above the rhizome (here denote as the second axillary bud, 2ndaxbud; see Pumisutapon *et al.* 2011, **Chapter 4** for a description of the sympodial growth of *Alstroemeria*) may generate a lateral rhizome, but stays under natural conditions usually dormant (Van Schaik 1998). The next upper axillary bud at the aerial shoot very seldom grows out, also when treated with cytokinin, and at the next higher nodes the formation of axillary buds itself is inhibited (Lin *et al.* 1998). The preferred method for large-scale

propagation is forced outgrowth of the 2ndaxbud (Buitendijk *et al*. 1992), despite its very low multiplication rate (1.2-1.8 per 4 weeks). So, strong apical dominance is a major problem in microprogation of *Alstroemeria*.

To improve micropropagation in *Alstroemeria*, elucidation of the mechanism(s) of apical dominance is necessary. In a previous study, we reported that outgrowth of the 2ndaxbuds is controlled not only by the shoot tip and but also by the rhizome tip. Auxin-lanolin application at the decapitated stumps restores apical dominance (Pumisutapon *et al*. 2011, **Chapter 4**). In this research, we investigate the effect of an SL analogue (GR24) and of SL biosynthesis inhibitors (fluridone and D2) on 2ndaxbud outgrowth and examine auxin transport in relation to the inhibition of SL synthesis.

Material and methods

Plant material and growth conditions

All experiments were carried out with the *Alstroemeria* hybrid '24098 2B' (Könst Alstroemeria, Nieuwveen, the Netherlands). The experiments of Figures 2 and 5 were also performed with the cultivar 'Sara' (Royal van Zanten, Rijsenhout, Netherlands). The standard medium for propagation of the stock and for all experiments was Murashige and Skoog (1962), 9 µM BAP, 4 % sucrose and 0.2% (w/v) Gelrite. In one experiment (Fig. 2), the BAP concentration was lowered to 3 µM BAP. The pH was adjusted to 5.8 before autoclaving (121°C for 20 min). The cultures were kept at 19°C and a 16-h photoperiod (30 µmol m^{-2} s^{-1}, TL 33 Philips, Eindhoven, The Netherlands). Plants were subcultured every 4 weeks by transferring rhizomes from which the larger part of the aerial shoots had been cut off (leaving *ca.* 1 cm aerial stem). Lateral rhizomes were separated from the main rhizome when sufficiently large (> *ca.* 1 cm).

Determination of 2ndaxbud outgrowth

The SL analogue GR24, the carotenoid biosynthesis inhibitor fluridone, the CCD biosynthesis inhibitor D2 and the auxin transport inhibitors NPA and TIBA were added to standard medium at the indicated concentrations. All growth regulators except BAP were

added after autoclaving. As explants, we used a rhizome with two shoots that were intact (+RhT,+2ShTs) or that had been removed for the larger part (+RhT,-2ShTs). The two aerial shoots were designated as Sh1 (located most far from the rhizome tip) and Sh2 (located close to the rhizome tip).

For each determination, five polystyrene jars (ø 66 mm) containing 25 ml medium with three explants each were used. Data were recorded after 4 weeks. The effects were evaluated with a χ^2-test. In the figures, means of 15 determinations ± SEs are shown. All experiments were repeated at least once.

Auxin transport assay

The assay was a modification of the protocol of C.J.M. Boot (Leiden University, pers. comm.). We used +RhT,-2ShTs explants that had grown for 6 weeks on standard medium or on medium with 3.6 µM fluridone or 3.6 µM fluridone plus 5 µM GR24. Segments of 10 mm excised from the main rhizome or from the aerial shoot were used. Petri dishes with a 5-mm layer of paraffin wax were prepared. In the wax, canals were cut: One apical canal, five basal canals, and five connecting canals between the apical canal and the five basal canals (Fig. 1). The five 10-mm segments were placed in the connecting canals (one segment in each). The apical cut ends of the segments were at the apical canal. Only in one determination, the basal cut ends were at the apical canal to check for acropetal transport. A mixture of lanolin and petroleum oil (3:1) was used for embedding (avoiding the cut ends) and for preventing leakage between the canals. After embedding the segments, liquid MS medium containing 1 µM IAA was added in the apical canal (4 ml) and basal canals (600 ml each). 5 µl of [^{3}H]IAA (185 kBq, American Radiolabeled Chemicals, specific activity 20 Ci mmol^{-1}, 1 mCi ml^{-1}) was added to liquid medium in the apical canal. After 8 h at room temperature in the dark (to prevent photooxidation of IAA) a sample of 500 µl liquid medium was collected from each canal and 4 ml Ultima Gold liquid scintillant (Perkin-Elmer Life and Analytical Sciences) was added. The amount of label was measured in a liquid scintillation counter. In the figure, means of 5 determinations ± SEs are shown. The statistical significance of differences was evaluated with a two-tailed Student *t*-test. The experiment was repeated twice.

To check for leakage, after the experiment all liquid was removed from the apical and basal canals (care was taken not to touch the stems) and a 0.05% (w/v) solution of acid fuchsin was pipetted in the apical canal. The Petri dishes were inspected after 1 h. When some dye solution had penetrated in a basal canal, this indicated leakage and the data from these canals were left aside. Leakage occurred in less than 5% of the connection canals.

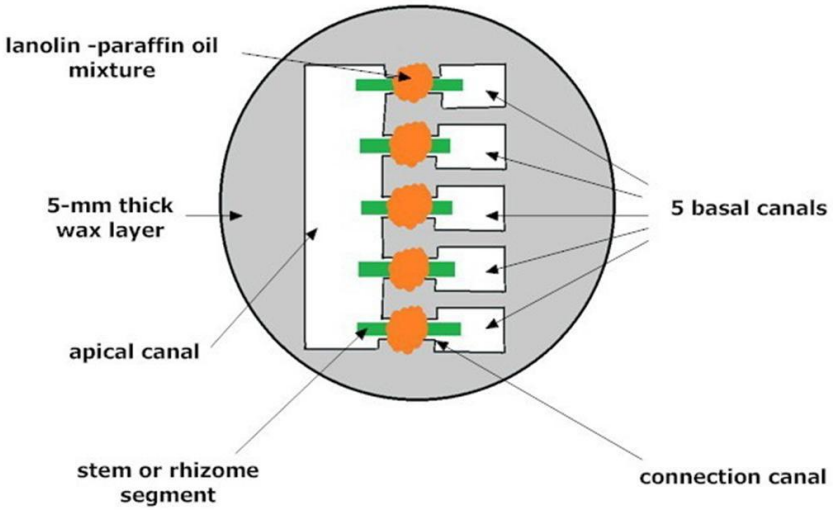

Figure 1. Petri dish with a paraffin wax layer and canals used to measure auxin transport in stem and rhizome segments of *Alstroemeria*.

Results

Effect of inhibitors in SL production/biosynthesis

We examined the effect of SL biosynthesis inhibitors on the outgrowth of the 2ndaxbud. We used fluridone (a carotenoid biosynthesis inhibitor) and D2 (an inhibitor of the oxidative cleavage of carotenoids and thereby of the synthesis of apocarotenoid signaling molecules). Figure 2 shows the promotive effect of fluridone at low BAP level (3 µM) on 2ndaxbud outgrowth in +RhT,+2ShTs explants of two *Alstroemeria* cultivars. In cultivar '24098 2B', the 2ndaxbud outgrowth at Sh1, Sh2 and the newly formed shoot Sh3

increased with the fluridone concentration. Almost all 2nd axbuds at Sh1 were released. The highest fluridone concentration (3.6 µM) gave the best result; the outgrowth of 2nd axbuds at Sh2 and Sh3 was increased by maximally *ca.* 35 and 15%, respectively. The outgrowth of 2nd axbuds in cultivar 'Sara' was also promoted by fluridone. 1.8 µM fluridone gave the best results. The outgrowth of 2nd axbuds at Sh1, Sh2 and Sh3 was increased by maximally *ca.* 40, 45 and 25%, respectively (for all, $P < 0.05$). Similar results were also obtained with +RhT,-2ShTs explants in both cultivars (data not shown). Even though fluridone caused bleaching, it did not reduce rhizome growth (data not shown). All fluridone-treated plants survived and they did recover from bleaching after subculturing on standard medium for a few cycles.

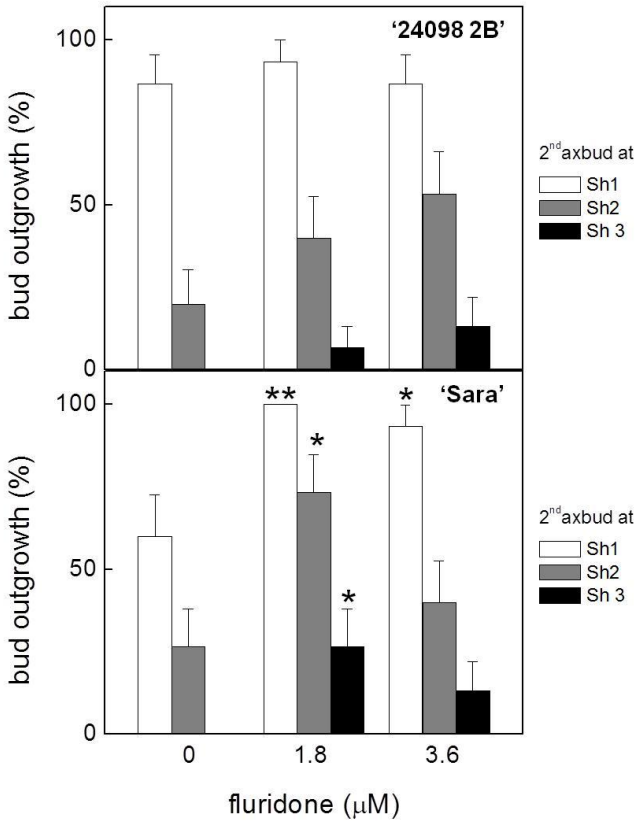

Figure 2. Outgrowth of 2nd axbuds at Sh1, Sh2 or a newly formed Sh3 in +RhT,+2ShTs explants of cultivars '24098 2B' and 'Sara'. Explants were grown for 4 weeks on medium containing 3 µM BAP and 0-3.6 µM fluridone.

The effect of D2 on 2nd axbud outgrowth in +RhT,-2ShTs explants is shown in Figure 3. Like fluridone, D2 enhanced the outgrowth of 2nd axbuds. The outgrowth increased with the D2 concentration up to 10 µM, and then decreased at higher concentration (50 µM). 10 µM D2 gave the highest 2nd axbud outgrowth in both Sh1 and Sh2 (30 and 20% increase, respectively; all treatments not significant different from control, but Sh1 at 10 µM $P < 0.07$).

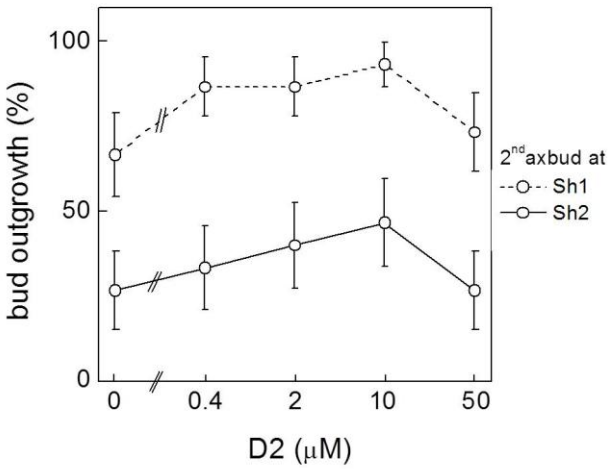

Figure 3. Outgrowth of 2nd axbuds at Sh1 or Sh2 in +RhT,+2ShTs explant. Explants were grown for 4 weeks on medium containing 9 µM BAP and 0-50 µM D2.

Effect of strigolactone

Application of the SL analogue GR24 inhibits bud outgrowth and reduces branching in pea and *Arabidopsis* (Brewer *et al.* 2009; Crawford *et al.* 2011). We investigated the effect of GR24 in *Alstroemeria*. The effect of GR24 alone or GR24 plus fluridone (3.6 µM) were examined in +RhT,+2ShTs explants. Figure 4 shows that increasing GR24 concentration decreased 2nd axbud outgrowth at Sh1, Sh2 and Sh3. The highest concentration (5 µM) reduced 2nd axbud outgrowth the most at Sh1 and Sh2 with 13 and 20% decrease, respectively (all not significant). At Sh3, GR24 completely inhibited 2nd axbud outgrowth. GR24 did not only inhibit 2nd axbud outgrowth but reduced rhizome growth as well (data not shown). When GR24 and fluridone were added together, fluridone

reduced the inhibition by GR24. This effect was larger on 2ndaxbud outgrowth at Sh2 than at Sh1. At the highest GR24 concentration (5 µM), fluridone improved 2ndaxbud outgrowth at Sh1 and Sh2 with 13 and 30%, respectively (all not significant).

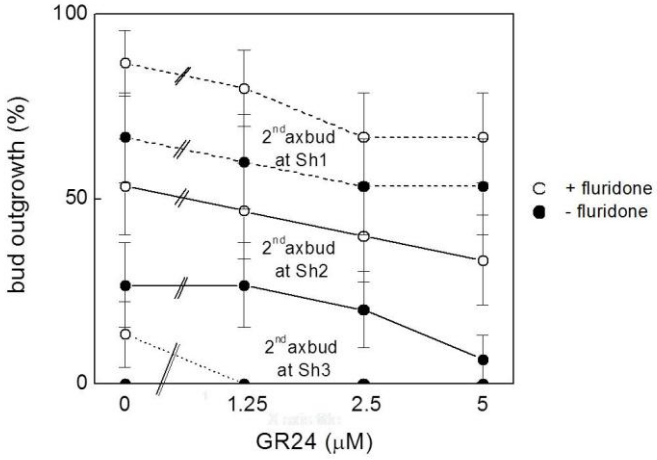

Figure 4. Outgrowth of 2ndaxbuds at Sh1, Sh2 or a newly formed Sh3 in +RhT,+2ShTs explants. Explants were grown for 4 weeks on medium containing 9 µM BAP and 0-5 µM GR24, either without or with 3.6 µM fluridone.

Effect of auxin transport inhibitor on promotive effect by fluridone

Auxin transport inhibitors usually increase the outgrowth of axillary buds. However, in the *Arabidopsis max* mutant NPA (an auxin transport inhibitor) decreased outgrowth of axillary buds suggesting that the observed increase of auxin transport in the *max* mutant caused increased axillary branching (Bennett *et al.* 2006). Possibly, in *Alstroemeria* the promotive effect by fluridone involves increased auxin transport. Therefore, we investigated the effect of auxin transport inhibitors on fluridone-promoted branching. First we examined the effect of NPA added to explants without fluridone (Fig. 5A). In both explant types, the outgrowth of 2ndaxbuds increased up to 11 µM NPA and then decreased at the higher concentration (22 µM). The effect was larger on 2ndaxbud outgrowth at Sh2 than Sh1. The highest increase of 2ndaxbud outgrowth at all shoot positions was at 11 µM NPA. The effect was significant ($P < 0.05$) for Sh1, +RhT,+2ShT explants and Sh2, both types of explant.

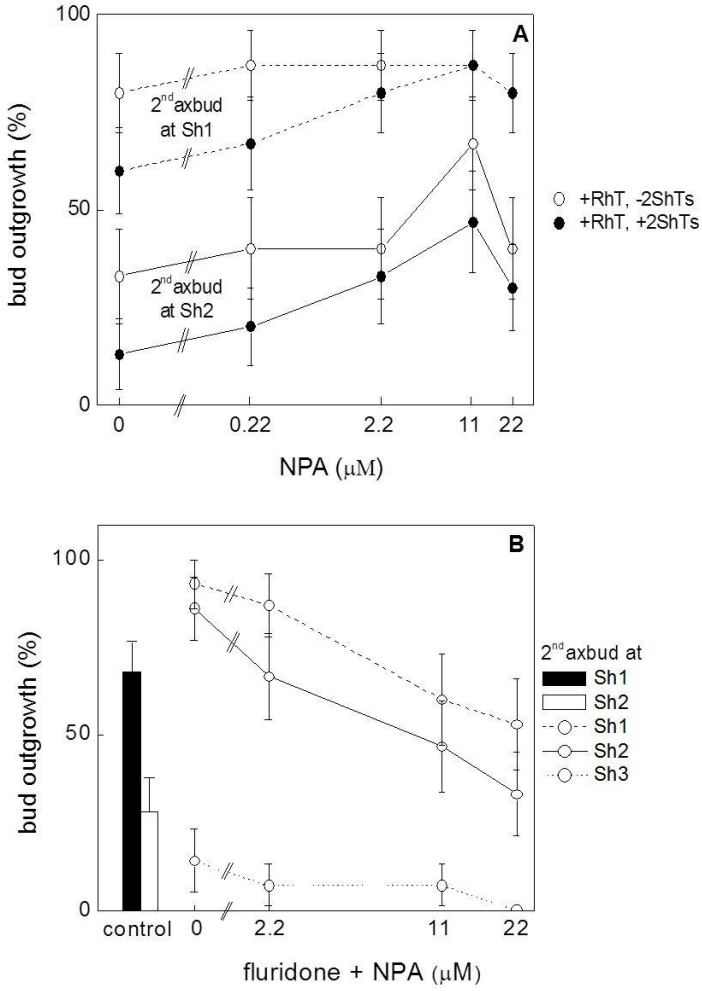

Figure 5. (A) Outgrowth of 2[nd]axbuds at Sh1 and Sh2 in +RhT,-2ShTs and +RhT,+2ShTs explants. Explants were grown for 4 weeks on medium containing 9 µM BAP and 0-22 µM NPA. (B) Outgrowth of 2[nd]axbuds at Sh1, Sh2 or a newly formed Sh3 in +RhT,+2ShTs explant. Explants were grown for 4 weeks on medium containing 9 µM BAP, 3.6 fluridone and 0-22 µM NPA.

Figure 5B shows 2[nd]axbud outgrowth in +RhT,+2ShTs explants when increasing NPA concentrations were added along with 3.6 µM fluridone. Fluridone alone significantly increased the growth of 2[nd]axbuds at all shoot positions. When NPA was added together

with fluridone, increasing NPA concentration reduced 2ndaxbud outgrowth compared to fluridone alone. NPA at 11 and 22 µM significantly reduced the promotive effect of fluridone on 2ndaxbud outgrowth at Sh1 and Sh2 (for all $P < 0.05$).

The same results were obtained with TIBA, another auxin transport inhibitor (2, 10 and 20 µM examined), and with the cultivar 'Sara' (data not shown).

Auxin transport

In the *Arabidopsis max* mutants, auxin transport is promoted (Bennett *et al.* 2006). We investigated whether fluridone increased auxin transport in *Alstroemeria*. Figure 6 shows the level of basipetal auxin transport in shoot and rhizome segments after 8 h.

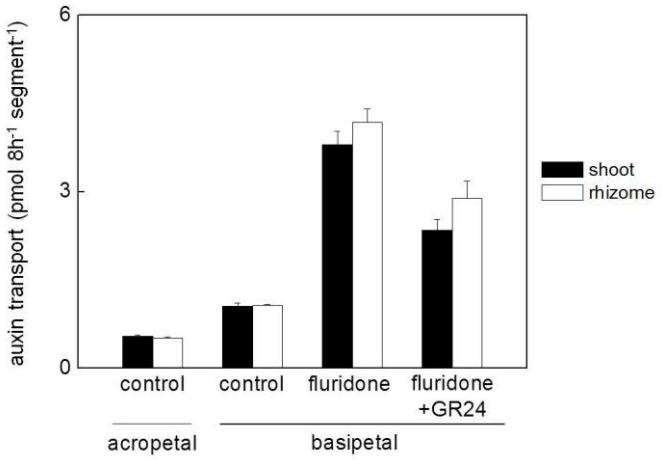

Figure 6. Auxin transport after 8 h in shoot and rhizome segments of the plants grown on the medium containing 9 µM BAP (control), 9 µM BAP plus 3.6 µM fluridone, or 9 µM BAP plus 3.6 µM fluridone and 5 µM GR24. The diameter of the explants was not changed by the hormonal treatment.

There was a large increase in auxin transport in shoot-stems (360%) and rhizomes (390%) of fluridone-treated plants compared to the control (for all $P < 0.001$). Plants treated with fluridone plus GR24 showed a significant decrease of auxin transport in shoot-stems (40%) and rhizomes (30%) compared to plants treated with fluridone alone (for

all $P < 0.05$). It should be noted that acropetal auxin transport also occurred in both shoot and rhizome and reached *ca.* 50% of basipetal auxin transport.

Discussion

SL inhibitors promote axillary bud outgrowth

We investigated whether inhibitors of SL biosynthesis did promote outgrowth of axillary buds (in this paper denoted as 2ndaxbud) in *Alstroemeria*. The inhibitors fluridone and D2 were added in the culture medium. Both increased axillary bud outgrowth. The effect of fluridone was relatively strong since it promoted outgrowth of axillary buds not only at Sh1 and Sh2 but also at the newly formed Sh3, even at a relatively low concentration (1.8 μM, Fig. 2). Apart from an earlier report (De Klerk 1992) this is the first report on enhancement of axillary bud outgrowth by fluridone. Fluridone is an herbicide inhibiting the biosynthesis of carotenoids. The enzyme phytoene desaturase is the target of fluridone. Because SL is synthesized from a carotenoid-precursor, this herbicide reduces endogenous SL production (Matusova *et al.* 2005). Fluridone is also known to inhibit the biosynthesis of abscisic acid, another carotenoid-derived hormone, which plays an important role in plant dormancy. Fluridone inhibits dormancy development and bulb formation in lily (Kim *et al.* 1994) and breaks dormancy in rose buds (Le Bris *et al.* 1999) and *Prunus campanulata* seeds (Chen *et al.* 2007).

Members of the CCD family split carotenoids at various chain positions, leading to the formation of a wide range of apocarotenoid signaling molecules. Compared to fluridone, the promotive effect of D2 on axillary outgrowth in *Alstroemeria* was not strong. It did not induce axillary bud outgrowth at Sh3, even at the most effective concentration (10 μM, Fig. 3). In *Arabidopsis*, the inhibitors of CCD7, CCD8, and/or MAX1 such as D2 stimulate shoot branching in stem sections and whole plants at the high concentration (100 μM), but the number of induced side branches is intermediate compared to the *max* branching mutants (Sergeant *et al.* 2009).

The role of SL in the inhibition of shoot branching has been investigated in several plants, *viz.*, in rice (Umehara *et al.* 2008), pea (Brewer *et al.* 2009) and *Arabidopsis* (Gomez-Roldan *et al.* 2008). Exogenously applied SL inhibits bud outgrowth and reduces

shoot branching (Umehara *et al.* 2008, Brewer *et al.* 2009, Crawford *et al.* 2010, Liang *et al.* 2010). We observed that application of the SL analogue GR24 to the culture media reduced axillary bud outgrowth in *Alstroemeria*. Furthermore, we found that fluridone rescued inhibited axillary buds of *Alstroemeria* when it was applied together with GR24.

Mechanism of SL action

The mechanism how SLs and auxin control of shoot branching is presently being debated. There is evidence supporting involvement of SL as a second messenger. The buds of SL mutants are resistant to apically supplied auxin (Beveridge *et al.* 2000, Sorefan *et al.* 2003, Bennett *et al.* 2006). In many cases, auxin upregulates the transcription of *CCD7* and *CCD8* genes (*e.g.*, Sorefan *et al.* 2003, Foo *et al.* 2005, Arite *et al.* 2007). Furthermore, grafting experiments suggest that branching in some auxin-signalling mutants is partly caused by SL deficiency (Hayward *et al.* 2009). These observations suggest that auxin moving in the polar auxin transport stream (PATS) upregulates SL biosynthesis, and that acropetally moving SL enters the axillary buds and represses their growth (Brewer *et al.* 2009). Correspondingly, direct application of SLs to the buds inhibits their outgrowth (Brewer *et al.* 2009). It is suggested that SL functions downstream of auxin in the main stem in its regulation of bud outgrowth in decapitated plants (Brewer *et al.* (2009).

An alternative hypothesis concerns action of SL via auxin transport. It has been proposed that axillary buds must export auxin into the PATS of the main stem to initiate bud activity (Bennett *et al.* 2006). It is thought that a shoot meristem, whether apical or axillary, can only grow out when it actively exports auxin into the PATS. In this model, SLs inhibit branching by reducing auxin transport, preventing the establishment of polar auxin export out of the axillary buds into the main stem, and thereby inhibiting their growth. Unlike the hypothesis mentioned above, SLs do not act only on local buds but they act systemically by modulating auxin transport (Bennett *et al.* 2006, Prusinkiewicz *et al.* 2009). Computational simulation supports that auxin transport is likely to proceed by canalization (Prusinkiewicz *et al.* 2009), the mechanism where the vascular strands are differentiated to connect auxin sources to sinks. It is consistent with subsequent observations of polarized accumulation of PIN auxin exporters during vascular strand patterning (Sauer *et al.* 2006). Furthermore, SL deficiency mutants which show increased

branching phenotype also have increased polar PIN accumulation in PATS and increased auxin transport (Bennett *et al.* 2006). These data correspond to the idea that SLs acts by limiting PIN accumulation, dampening canalization and thus preventing bud outgrowth.

As mentioned before, fluridone increased axillary bud outgrowth in *Alstroemeria*. We were interested whether fluridone influenced auxin transport. In a previous study, we have reported that the auxin transport inhibitor TIBA increases axillary bud outgrowth in *Alstroemeria* (Pumisutapon *et al.* 2011, **Chapter 4**). This is consistent with the idea that if the level of auxin transport is reduced, branching is promoted (Bennett *et al.* 2006). Fluridone-treated plants were given increased concentrations of auxin transport inhibitors (NPA and TIBA) to find out whether fluridone-treated plants would respond in the same way as *Arabidopsis* SL mutants. Both auxin inhibitors reduced axillary bud outgrowth of *Alstroemeria* treated with fluridone. This indicates that the increase in axillary bud outgrowth by fluridone is brought about by increased auxin transport.

In an auxin transport assay, there was a marked increase in basipetal auxin transport in shoot-stems and rhizomes excised from *Alstroemeria* plants grown in the presence of fluridone. This also indicates involvement of increased auxin transport in the promotion of axillary bud outgrowth. Fluridone apparently acts similar to *max* genes to inhibit SL biosynthesis, subsequently increase PIN accumulation in PATS and thus increasing auxin transport. Furthermore, GR24 reduced auxin transport in shoot and rhizome even when *Alstroemeria* plants received fluridone. This is consistent with the finding in *Arabidopsis* that GR24 is able to reduce auxin transport in wild type stems and PIN accumulation in xylem parenchyma cells in wild type and SL mutant, and it is suggested that SLs act systemically by dampening auxin transport (Crawford *et al.* 2010).

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Chapter 6

General Discussion

Micropropagation via axillary-bud branching is the most widely used method for commercial micropropagation, because it produces true-to-type plantlets (there are some exceptions, see **Chapter 1**), is reliable with respect to production numbers and requires a lower level of expertise. However, this method usually gives lower multiplication rates compared to other methods because of the limited number of axillary buds that actually grow out. This also holds true for *Alstroemeria*, a crop which is commercially micropropagated via rhizome branching, which is achieved by forced outgrowth of the second axillary bud of the vertically growing shoot. This method gives a very low multiplication rate (less than 2 per cycle of 4 weeks) because of strong apical dominance and slow growth of rhizomes. With respect to the latter, it should be noted that the growth of lateral rhizomes is often so slow that they cannot be separated from the main rhizome at the end of the subculture cycle but only after a following subculture cycle. All these are the major obstacles in micropropagation of *Alstroemeria*. The aim of this thesis is to improve micropropagation of *Alstroemeria* by studying the basic and applied aspects of the following main topics: (1) nutrient consumption during a micropropagation cycle as related to rhizome growth, (2) moderate abiotic stress and its effect upon rhizome growth and (3) apical dominance mechanisms in the control of axillary bud outgrowth.

Approaches to enhance rhizome growth

It would be a great benefit when the main rhizome and lateral rhizomes grow better so that they can be separated sooner during subculturing. We studied two main topics to find new approaches to enhance rhizome growth: nutrient consumption during a micropagation cycle (**Chapter** 2) and application of moderate abiotic stresses (**Chapter** 3).

Nutrient media used in plant tissue culture supposedly contain all the components necessary for growth *in vitro*: inorganic nutrients, sucrose as an energy source and as a source of building blocks, plant hormones and vitamins. Understanding the mechanisms of movement of solutes in tissue culture may offer new possibilities for enhancement of growth *in vitro* (De Klerk 2010). Particular minerals may have an important role in supporting growth as well as generally regulating development. The rate of mineral uptake for a specific ion may exceed its rate of utilization. Pools of available ions may form within the explant allowing continued growth well after the uptake of the ion

has ceased. Understanding the nutritional requirements of a culture system is necessary for optimal growth of cultured plantlets. Nutrient consumption has mostly been studied in liquid culture systems (Leiffert *et al.* 1995) including in *Alstroemeria* (De Klerk and Ter Brugge 2010). Detailed studies in solidified medium though are relatively rare. In **Chapter 4**, we studied nutrient depletion in solidified medium which is normally used for commercial micropropagation of *Alstroemeria*. Inorganic nutrients were taken up faster than sucrose, possibly due to the higher diffusion coefficient of inorganic nutrients and different rates of active uptake by the explants. A large portion of nutrients still remained at the end of a subculturing cycle (4 weeks). This suggests that it is possible to reduce the nutrient concentration in the culture medium or to extend the subculture cycle. Furthermore, individual inorganic nutrients were taken up at different rates. The characterization of ion uptake depends on genotype and selectivity. Certain mineral elements are taken up preferentially, while others are discriminated against or almost excluded (Marschner 1995). Our observations showed that the order of inorganic-nutrient exhaustion was similar to a previous study in liquid medium (De Klerk and Ter Brugge 2010) and to other cases where tissues show fast growth (Schmitz and Lörz 1990). This suggests that it is unlikely that slow growth in *Alstroemeria* is caused by exhaustion of specific nutrients. Therefore adaptation of medium composition and concentration according to the order of exhaustion may not be effective to enhance the growth of *Alstroemeria*.

Elemental analysis of plant tissues provides information on distinctive elemental compositions of plant species. This information is beneficial for adaptation of nutrient formulation. Several formulations have been developed based on tissue analysis and used successfully. For example, Staikidou *et al.* 2006 developed the medium based on mineral analysis of storage organ bulbs in *Galanthus* species and found that this medium increased bulblet initiation and supported bulblet growth and rooting better than MS medium. When we determined inorganic composition in tissues of *Alstroemeria*, there were marked differences between rhizome and aerial tissues in the levels of some macro-elements **(Chapter 4)**. Mg and P were relatively low and Ca was relatively high in rhizomes. We hypothesized that lower Mg and P and higher Ca in the culture media might increase rhizome growth. Only adaptation of Mg improved rhizome growth a little. However, adaptation of P and Ca did not improve rhizome growth. In other crops with storage organs

(*e.g.*, bulbs: tulip and lily), adaptation based on endogenous elementary levels also did not bring about improved growth (H Bouman, Bulb Research Centre, Lisse, the Netherlands pers. comm.). This suggests that adaptation of specific nutrients in order to enhance the growth of plant storage organs seems to be rather difficult to accomplish. Otherwise, the experimental procedure should be improved, for example, use more appropriate chemicals (*e.g.*, $Ca(NO_3)_2$ instead of $CaCl_2$; chloride is toxic at high concentration) and use the formulation which contains low Mg and P and high Ca levels altogether instead of adjustment separately (see *e.g.*, Bouman and Tiekstra 2005, Staikidou *et al.* 2006).

In natural and agricultural conditions, plants often grow under unfavorable conditions leading to abiotic stresses: heat, drought, salinity, cold, water logging, radiation, oxidative stress, mineral deficiency and mineral toxicity. These abiotic stresses can delay growth and development, reduce productivity and, in extreme cases, cause death of the plants. To ensure their survival and the prosperity of their offspring, plants have developed a range of mechanisms to cope with various abiotic stresses. Plants have developed tolerance mechanisms during unfavorable environmental conditions in order to withstand the stress. A tolerance mechanism activated by different abiotic stresses is accumulation of protective low-molecular-weight compounds (Bohnert *et al*. 1995) *viz.*, proline, glycine betaine, polyamines or trehalose, and protective proteins, *viz.*, heat shock proteins (HSPs; chaperone-function, Wang *et al.* 2003). Direct application of low-molecular-weight protectants results in resistance to stress (Bae *et al*. 2005, Zeid and Shedeed 2006, De Klerk and Pumisutapon 2008). When plants are exposed to moderate stress, they may initiate protective mechanisms to resist future severe stress (Larkindale *et al*. 2005, De Klerk and Pumisutapon 2008). Furthermore, many avoidance mechanisms which prevent exposure to stress have been developed during evolution *e.g.*, sunken stomata, light reflective spines and deep roots. These traits are genetically determined and are expressed whether the plants are stressed or not. Plants also develop dormancy and generate storage organs to survive predictable climatic stresses and to ascertain fast regrowth after that. Plants that grow under stressful conditions tend to allocate a high proportion of biomass to below-ground biomass (roots and storage organs) compared to that above-ground (Fritz *et al.* 2004, Puijalon *et al.* 2005), but severe stress reduces the growth of storage organs (Ewing 1981, Chang and Randle 2004).

In **Chapter 3**, we investigated the effect of various types of moderate abiotic stress namely heat (hot-air and hot-water treatments), cold (0ºC), anaerobiosis, drought and salinity on the growth of *Alstroemeria* rhizomes. All resulted in increased rhizome growth. It seemed that moderate stress specifically increased the sink activity of the rhizome because shoot growth after the stress did not increase. The increase of growth is likely based on the protective capabilities of the rhizome. Under the stress conditions applied, heat stress gave high promotion on rhizome growth as well as rhizome propagation. Cold stress also gave satisfactory results. Thus, moderate stress is a promising tool to enhance rhizome growth of *Alstroemeria* cultured *in vitro* and may be applied to other crops with storage organs such as tubers, bulbs, corms and rhizomes. However, the after-effects of moderate-stress treatments should be evaluate and the protocols may be adjusted before using commercially.

Recent theories in regulation of apical dominance

Since strong apical dominance causes a low multiplication rate in *Alstroemeria* both *in vivo* and *in vitro*, and because of recent discoveries about the regulation of apical dominance, a large part of this thesis concerned the study of apical dominance mechanism(s) in *Alstroemeria* (**Chapters 4** and **5**). Here, we give a brief overview on the conventional and the newer theories on apical dominance mechanisms and describe below how our research could be linked to these theories.

The outgrowth of axillary buds is partially or totally inhibited by the shoot apex. Apical dominance is the control exerted by the shoot apex over the outgrowth of axillary buds. This developmental phenomenon is a plant survival mechanism and provides a reservoir of meristems that can replace a damaged primary shoot. On the other hand, without apical dominance, plants will be extremely bushy and short leading to a reduced ability to capture light for photosynthesis and thereby to reduce fitness. The development of apical dominance and its release can be divided into four stages: axillary bud formation (stage I), imposition of inhibition (apical dominance; stage II), initiation of axillary bud outgrowth following decapitation (stage III) and subsequent elongation and development of the axillary bud into a branch (stage IV) (Cline 1997). With respect to hormonal effects, the role of the conventional plant hormones in different stages may be defined as follows:

cytokinin promotes (stage I), auxin represses (stage II), cytokinin releases (stage III) and auxin and gibberellin promote (stage IV). Axillary buds originate from the growing shoot apex (stage I). An axillary meristem is typically located in the leaf axils. It is formed from the detached part of the primary shoot apical meristem and leaf primordium and becomes organized into the apex of the axillary bud. In some plants, axillary meristems undergo immediately development to form visible axillary shoots (Stage IV). In many plants, axillary meristems may initiate a few leaves and then become developmentally arrested or dormant because the apical bud inhibits the outgrowth (stage II). These dormant axillary buds resume development at later time depending on their developmental programme or in response to environmental conditions (Shimizu-Sato and Mori 2001). Although the inhibited axillary buds are non-growing buds, they still are metabolically active (Stafstrom and Sussex 1992, Leyser 2003). Once the shoot apex is decapitated, apical dominance is released (stage III). Subsequently, one or more inhibited axillary buds can grow out. The release of apical dominance may be promoted by direct application of cytokinin to the axillary buds. On the other hand, application of exogenous auxin to decapitated stump represses this release.

Even though much of the mechanism of apical dominance is known, the precise regulation remains unclear. Regarding hormonal signalling, auxin and cytokinins are conventionally thought to be the major signals in the regulation of apical dominance and axillary branching. Auxin derived from the apical bud has an inhibitory effect on the axillary bud outgrowth, whereas cytokinin derived mainly from the nodal stem as well as from the root promotes the growth of axillary buds. Auxin produced by the primary shoot apex is transported basipetally in the shoot. Initially, it was believed that auxin transports basipetally down the stem and then moves upwards reaching the axillary buds where it would directly inhibit their growth (Thimann and Skoog 1933, Cline 1994). However, this hypothesis has been reputed, because monitoring translocation of radio-labeled auxin shows that auxin does not move acropetally into the branches (Shimizu-Sato and Mori 2001, Leyser 2005). This indicates that auxin does not work directly in controlling bud outgrowth and leads to the hypothesis that auxin acts indirectly through the action of upward-moving second messenger(s) (McSteen and Leyser 2005, Leyser, 2005).

The proposed candidates which could act as upward-moving second messenger(s) for auxin-mediated axillary bud inhibition are cytokinins and strigolactones

(SLs) (McSteen and Leyser 2005, Leyser, 2005). In this model, auxin represses local biosynthesis of cytokinins in the nodal stem. Decapitation increases cytokinin biosynthesis in the nodal stem and cytokinin translocation to axillary buds results in promotion of bud outgrowth (Dun *et al.* 2006, Tanaka *et al.* 2006). Auxin may act by limiting the amount of cytokinins that are translocated into the bud. It can down-regulate cytokinin biosynthesis in the stem and cytokinin export in the xylem from the root. In addition, an auxin-cytokinin gradient may control the axillary bud outgrowth. If the ratio changes in favor of cytokinins, the axillary buds are released from inhibition. If the ratio increases the inhibition will be maintained (Srivastava 2002).

Recently, carotenoid-derived SLs have been proposed as branching inhibiting hormones (Gomez-Roldan *et al.* 2008, Umehara *et al.* 2008, Domagalska and Leyser 2011). SLs are synthesized in both roots and stem and transported upward in the xylem (Beveridge 2006). Biosynthesis of SLs depends on the activity of the *MORE AXILLARY GROWTH (MAX) 1*, *3* and *4* genes in *Arabidopsis*, the *RAMOSOUS* (*RMS*) *1* and *5* genes in pea, the *DECREASE APICAL DOMINANCE 1* (*DAD1*) genes of petunia and the *DWARF (D) 10* and *17* or *HIGH-TILLERING DWARF (HTD) 1* genes in rice (Beveridge *et al.* 2009, Beveridge and Kyozuka 2010, Dun *et al.* 2009, Leyser 2008, 2009). For all these genes, mutants with a loss of function show an increased branching phenotype (McSteen and Leyser 2005, Leyser 2005, Beveridge 2006, Umehara *et al.* 2008). In the biosynthesis pathway, *MAX3*, *RMS5 and HTD1/D17* encode CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), whereas *MAX4*, *RMS1*, *D10* and *DAD1* encode another subclass of CCDs designated as CCD8. CCD7 and CCD8 might catalyse sequential carotenoid cleavage reactions (Booker *et al.* 2004). *MAX1* is a cytochrome P450 monooxygenase presumably involved in a later biosynthetic step (Booker *et al.* 2005). Furthermore, *MAX2*, *RMS4* and *D3* encode F-box protein and are involved in signal transduction of the novel hormone (Johnson *et al.* 2006, Stirnberg *et al.* 2002, Ishikawa *et al.* 2005).

For the mode of action of SLs and their relation with auxin, two hypotheses have been put forward. According to the first, auxin moving in the main stem influences SLs by upregulating SL biosynthesis. Acropetal moving SLs enter the axillary buds and inhibit their growth. So, SLs act as second messengers for auxin, relaying the inhibitory signal from the main stem into the bud (Brewer *et al.* 2009, Hayward *et al.* 2009).

An alternative theory has been proposed as the auxin transport hypothesis. SLs are presumed to control bud outgrowth via auxin transport. Supposedly, there is a competition between apical and axillary buds to export auxin into the polar auxin transport stream (PATS) in the main stem. This export is required for bud outgrowth. The competition results from a limited capacity of the main stem to transport auxin (Bennett *et al.* 2006). In this hypothesis, SLs act systematically by determining the rate of auxin transport. It is proposed that SLs down-regulate PIN auxin exporters, thus causing decreased auxin transport capacity in PATS. This would prevent the axillary buds to grow out because they cannot export auxin into the PATS of the main stem (Bennett *et al.* 2006, Domagalska and Leyser 2011). The increased branching phenotype of SL *Arabidopsis* mutants correlates with increased PIN accumulation and increased auxin transport capacity in PATS (Bennett *et al.* 2006).

Axillary buds may response to a range of signals differently depending on their developmental stages. The bud transition hypothesis proposes that the bud enters different developmental stages having varying degrees of sensitivity or responses to long-distance signals, including auxin (see review in Dun *et al.* 2006). In this hypothesis, existing buds may reside in at least three stages: a stage of dormancy, a stage of transition, or a stage of sustained growth. Here, the term dormancy is referred to the extremely low growth rate of the axillary buds despite the fact that these buds are metabolically active. In the transition stage(s), axillary buds are more responsive than dormant buds to the signals that stimulate their growth, yet remain able to revert to a stage of dormancy. Various factors are involved in the determination of the developmental stage of a bud: the stage of whole-plant ontogenetic development, the particular node at which the bud arises, the age of the bud, genotype and environmental factors (*i.e.*, light, temperature, and photoperiod). In different situations, non-growing buds have different responses to various treatments that may stimulate bud outgrowth, it is useful to consider that bud outgrowth consists of several stages and that particular signals may act at some, but not all, of these stages. Furthermore, a treatment such as decapitation causes a rapidly propagated signal that triggers a dormant bud to enter a transition stage. In this transition stage, the bud starts preliminary growth only (Morris *et al.* 2005). If the bud transition hypothesis is incorporated with the classical hypothesis, auxin may act to inhibit progression of buds from a transition stage to sustained bud outgrowth by modulating local cytokinin biosynthesis, and possibly cytokinin transport,

and/or by exerting its regulation of SLs. Similarly, if auxin transport capacity is the limiting factor, then it may be important at a transition stage (Dun *et al.* 2006).

Apical dominance in *Alstroemeria*

In **Chapter 4**, we studied apical dominance in *in vitro*-grown *Alstroemeria*, starting off from the classical theory. By decapitation experiments, we found that the outgrowth of axillary buds (= 2ndaxbuds) was controlled by both shoot tips and rhizome tips (= 1staxbud), albeit with different intensity. The rhizome tip had stronger influence on axillary bud outgrowth than the shoot tip. The effect of removal of both tips was additive. The effect of basipetally transported auxin on inhibition of axillary bud outgrowth was demonstrated by classical auxin-lanolin application (Thimann and Skoog 1933) to excised shoot and/or rhizome tips and the applications of auxin transport inhibitors (TIBA and NPA). We found that replacement of these tips by IBA-lanolin restored apical dominance, whereas both TIBA and NPA released inhibited axillary buds. Application of cytokinins (BAP and TDZ) promoted axillary bud outgrowth. All these observations implicated that auxin inhibited and cytokinin promoted axillary bud outgrowth in *Alstroemeria*, which is consistent to the classical hypothesis of apical dominance.

In Chapter 5, we focused on the role of the novel branching inhibitors SL. Similar to the observations in model plants (Umehara *et al.* 2008, Brewer *et al.* 2009, Crawford *et al.* 2010, Liang *et al.* 2010), exogenous application of a SL analogue (GR24) reduced axillary bud outgrowth. Several researchers report that the mutants in SL biosynthesis have an increased branching phenotype (review in Domagalska and Leyser 2011), possibly caused by increased auxin transport capacity of the main stem (Bennett *et al.* 2006, Ongaro and Leyser 2008). However, a limitation in this thesis was that SL mutants of *Alstroemeria* are not feasible. Therefore, we treated *Alstroemeria* plants with SL biosynthesis inhibitors to examine whether this leads to increased axillary bud outgrowth, similarly to SL mutants. We applied the carotenoid biosynthesis inhibitor fluridone and the CCD biosynthesis inhibitor D2 to the plants. Both inhibitors increased axillary bud outgrowth. According to the auxin transport hypothesis, SLs inhibit axillary bud outgrowth by preventing the axillary buds to export their auxin into the PATS of the main stem because SLs down-regulate PIN auxin efflux carriers and this causes limited auxin transport

capacity in PATS (Bennett *et al.* 2006, Ongaro and Leyser 2008). Auxin transport inhibitors (TIBA and NPA) increased axillary bud outgrowth when added alone (**Chapter 4**). However, axillary bud outgrowth was reduced by the transport inhibitors when we treated *Alstroemeria* plants grown in the presence of fluridone with TIBA or NPA. With an auxin transport assay, we found a marked increase in basipetal auxin transport level in both shoots and rhizome of fluridone-grown plants. All these evidences suggested that SLs inhibited axillary bud outgrowth in *Alstroemeria* via manipulation of auxin transport.

Even though our results in **Chapter 5** were exactly in line with the auxin transport hypothesis that SLs act systematically by modulating auxin transport, we should not ignore the classical concept that SLs act only locally in the inhibition of axillary bud outgrowth. This can be investigated further by direct application of SLs to the buds (Brewer *et al.* 2009). In addition, we found that axillary buds of shoots located at different positions on the rhizome showed different levels of inhibition (**Chapters 4** and **5**). In most cases, axillary buds at the shoot located farthest from the rhizome tip such as Sh1 always grew out (almost 100%), whereas axillary buds at shoots located close to the rhizome tip such as Sh2 and newly formed Sh3 grew out occasionally (< 60%) or hardly (0%), respectively. This may involve developmental stages of the axillary bud according to bud transition hypothesis. The axillary buds at the younger shoots (Sh2 and Sh3) may be in a dormant stage, thus respond to a stimulation signal less than the ones at the older shoot (Sh1) which may already have proceeded to the transition stage.

Approaches to achieve axillary bud outgrowth in micropropagation

The economic implications of apical dominance in agricultural and ornamental species are substantial. As mentioned in **Chapter 1**, micropropagation by axillary branching is the most reliable method among the various procedures because it produces uually true-to-type plantlets. Two methods are commonly used: shoot culture and node culture. Both methods depend on stimulating precocious axillary shoot growth by overcoming apical dominance exerted by shoot apical meristems (George and Debergh 2008). Shoot (or shoot tip) culture is started from explants bearing an intact apical shoot meristem. By the repeated formation of axillary branches propagation is achieved. This is the most widely used method of micropropagation. Single and multiple node culture is

another *in vitro* propagation technique in which each shoot may be cut into single- or several node pieces which are subcultured, so that each explant consists of a piece of stem bearing one or more lateral buds. It is the best propagation method for plants that fail to produce multiple shoots at Stage II (see **Chapter 1**) and retain their apical dominance. Node culture is important for propagating species that produce elongated shoots (*e.g.*, potato, Hussey and Stacey 1981, Kristensen 1984) especially if stimulation of lateral bud break is difficult to bring about with cytokinins.

To achieve forced outgrowth of axillary buds *in vitro*, several approaches based on the conventional concept of hormonal regulation of apical dominance, have been used. The growth and proliferation of axillary shoots is usually promoted by incorporating growth regulators (usually cytokinins) into the growth medium. Physical manipulation such as decapitation is also used to remove the dominance of apical meristems, so that axillary buds can grow out and axillary shoots are produced. Cytokinins are usually extremely effective in removing the apical dominance of shoots. In micropropagation, cytokinins are applied to promote axillary branching. Their use can be combined with decapitation of shoot apices. Several conventional cytokinins are commonly used *in vitro* including benzylaminopurine (BAP or benzyladenine, BA), zeatin, kinetin and 2iP. In some cases, relatively 'new' cytokinins, for example, thidiazuron (TDZ), 4PU and meta-topolin are much more effective than the conventional ones (Genkov and Ivanova 1995, Bairu *et al.* 2007, Khurana-Kaul *et al.* 2010). In *Alstroemeria*, though we found that TDZ did not give better result than BAP in improvement of axillary bud outgrowth (**Chapter 4**). In addition, auxin transport inhibitors (*e.g.*, TIBA) are occasionally used (Singh and Syamal 2000). The growth regulators efficiently promote axillary bud outgrowth. However, they may cause undesirable side-effects, for example, cytokinins induce hyperhydricity and cause the loss of the chimeric structure, and high concentrations of cytokinin can lead to extreme bushiness (George and Debergh 2008).

In this thesis, we also used conventional methods and found that such applications promote the growth of axillary buds (**Chapter 4**). Decapitation of both shoot and rhizome tips gave the best axillary bud outgrowth. However, this method is not acceptable for commercial micropropagation because the main rhizome is irreversibly damaged. By using the explants with an intact rhizome tip, new aerial shoots are produced by continued growth of the rhizome tip. These new shoots provide new axillary buds (one

2nd axbud per shoot) that can be 'used' in the next subculturing cycle to achieve branching. Application of cytokinins (BAP and TDZ) and auxin transport inhibitors (TIBA and NPA) to the culture medium could promote axillary bud outgrowth but the effect was not very large. This suggests that application protocols may not suitable or apical dominance in *Alstroemeria* is too strong. It seems that these conventional approaches may not be sufficient to improve axillary bud outgrowth and propagation factors in *Alstroemeria.*

In **Chapter 5**, we tried a new approach which was application of SL biosynthesis inhibitors (fluridone and D2). Both inhibitors were able to promote axillary bud outgrowth. The effect of D2 was not strong. Fluridone gave satisfactory improvement and it also could release axillary buds at the newly formed shoots (*e.g.,* Sh3) which had never happened by applications of the conventional methods mentioned above. Furthermore, fluridone did not cause irreversible damage and did not reduce growth. Bleaching after fluridone treatment disappeared by transferring the plants to standard propagation medium for a few cycles. Therefore, the use of fluridone seems to be a promising approach to improve the propagation factor in *Alstroemeria*. In addition, it is possible to apply fluridone as pulse treatment. This method allows using fluridone at high concentration which may give more effective results and may reduce the appearance of bleaching because the plants receive fluridone only for a very short period.

Conclusion and future prospects

Alstroemeria has low natural propagation rates due to restricted development of axillary meristems. It has in fact only two axillary buds on each aerial shoot, one is the rhizome apex which always grows out and continues the rhizome. The next higher one (the 2nd axbud) has the potential to grow into a lateral rhizome. The initials of axillary buds or meristematic structures do not exist at the leaf axils of the aerial shoot-stems (Lin *et al.* 1998), indicating that this plant has strong apical dominance. In *Alstroemeria* micropropagation, the preferred method is rhizome propagation which is achieved by forced outgrowth of the second axillary bud located just above rhizome tip. However, this method still gives low propagation factor also because the outgrowth of the lateral rhizomes is generally slow. Increases in growth and multiplication of rhizomes are highly required

since slow growth and low multiplication rate of rhizomes are the main obstacles for commercial production.

In this research, we examined the effects of nutrients and moderate stresses to improve rhizome growth and apical dominance to improve rhizome propagation. Adaptation of specific nutrients in culture medium based on elemental compositions in plant tissues did not bring about significant improvement, indicating that enhanced rhizome growth is unlikely influenced by specific components. Application of moderate abiotic stresses, *viz.*, heat and cold, effectively enhanced the growth of the rhizome (more than 70%). Increased rhizome growth is a protective response activated by moderate stress. Thus, moderate stress is a promising approach to increase rhizome growth in *Alstroemeria*. In future study, it is worth to examine the other plausible methods which induce plant storage organ formation, *e.g.*, the uses of growth retardants and high sugar level.

In the study on apical dominance, we demonstrated that the outgrowth of the axillary bud which develops into a lateral rhizome was controlled by both rhizome and shoot tips. Decapitation of both tips released the axillary buds. Replacement of excised tips by auxin-lanolin paste restored apical dominance, indicating an inhibitory effect of auxin in the control of axillary bud outgrowth. Auxin transport inhibitors and cytokinins promoted axillary bud outgrowth but the effect was not strong. Application of the SL analogue GR24 reduced outgrowth of axillary bud. The inhibitor of SL biosynthesis that highly promoted axillary bud outgrowth was fluridone. Increased axillary bud outgrowth by fluridone treatment involved auxin transport since it was reduced by auxin transport inhibitors. This evidence indicates that SL acts via inhibition of auxin transport. In an auxin transport assay, there was also a marked increased of basipetal auxin transport in shoot-stems and rhizomes (350-400%) of plants cultured with fluridone, suggesting that increased auxin transport promotes of axillary bud outgrowth. In this study, SLs played a role in the control of axillary bud outgrowth of *Alstroemeria* by regulating auxin transport, similarly to effect observed in model plants. Application of inhibitors of SLs biosynthesis such as fluridone is useful in plant propagation where the plants exhibit strong apical dominance and in production of plants with compact architecture *e.g.,* in orchard crops. Study of apical dominance in *Alstroemeria* can be the model for rhizomatous plants. In the future, increased axillary bud outgrowth of *Alstroemeria* could be efficiently achieved by

application of SL biosynthesis inhibitors such as fluridone and the after-effects should be evaluated before commercial use.

Finally, we have summarized the approaches to improve propagation factors in *Alstroemeria* micropropagation in Figure 1.

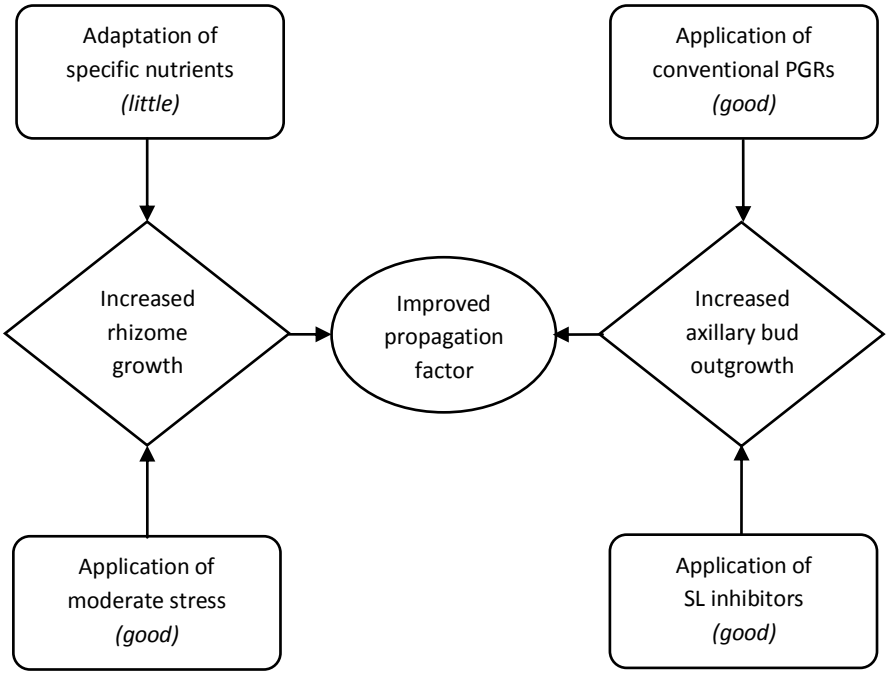

Figure 1. Scheme summarizing the approaches to improve the propagation factor of *Alstroemeria* cultured *in vitro*. In brackets is an estimation of the level of success.

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Summary

Alstroemeria is a high-value ornamental plant. Hybrids are used mainly for cut flower production and as potted plants. The growth habit is sympodial. At a node, the rhizome apex changes direction of growth from horizontal to vertical and forms a new aerial shoot. The axillary bud at this node always grows out horizontally becoming the new rhizome apex, and forms the next internode of the rhizome. At the next node, the rhizome apex again changes direction of growth to vertical and forms a new aerial shoot. The axillary bud at this node develops horizontally as the previous one forming an internode of the rhizome. This process repeats itself. Thus a rhizome consists of a chain of basal internodes of successively developed shoots.

The second axillary bud at the aerial shoot locates just above the first one. It usually stays dormant because of strong apical dominance but occasionally develops into a lateral rhizome when the aerial shoot dies or is removed. No further axillary buds appear at the axils of higher leaves. Thus *Astroemeria* has low natural propagation rates due to restricted development of axillary meristems. Micropropagation is the preferred method for commercial production of *Alstroemeria*. It involves the formation of lateral rhizomes brought about by forced outgrowth of the second axillary buds of the vertically growing shoot. However, this method still gives a low propagation factor (usually far less than 2 per cycle). This is caused by a combination of strong apical dominance and poor rhizome growth.

In this thesis, the general aim was to improve micropropagation by enhancing growth and multiplication of *Alstroemeria* rhizomes. Furthermore, another more scientific aim was focused on basic and applied studies of the following aspects: (1) obtain insight into the nutrient consumption during a micropropagation cycle as related to the growth of rhizomes; (2) the influence of moderate abiotic stress and its effect on rhizome growth and, (3) investigating apical dominance mechanism(s) on the controlled outgrowth of axillary buds.

Poor rhizome growth is one of the major obstacles in *Alstroemeria* micropropagation. For propagation, the size of lateral rhizomes should be large enough, so they can be separated from the main rhizome as soon as possible. Nutrient consumption was studied during *in vitro* culture to improve rhizome growth. 10 and 25 ml solid MS media were compared over a period of 4 weeks. Both rhizome growth and multiplication were higher in 25 ml medium. The same quantity of sucrose was taken up in 10 ml and 25

ml but the quantity of inorganic nutrients taken up in 25 ml was twice as high as that in 10 ml. The observed differences between the two volumes can likely be explained by different diffusion rates of components and the thickness of the medium so that in 10 ml the diffusion-distances to the explants are larger. NH_4^+ and $H_2PO_4^-$ were taken up most rapidly, and Mg^{2+} and Ca^{2+} were still at a high concentration after 4 weeks. According to elemental analysis of tissue-cultured plants, Mg and P levels were relatively low, and Ca level was relatively high in rhizomes compared to shoots and leaves. We hypothesized that decreasing Mg and P, and increasing Ca concentrations in the culture medium might improve rhizome growth. However, only reducing Mg^{2+} to half standard level of MS formulation improved rhizome growth (*ca.* 30%). When $H_2PO_4^-$ concentration was decreased or Ca^{2+} concentration was increased compared to the control, rhizome growth was not improved, suggesting that enhanced rhizome growth is unlikely influenced by specific components.

When plants are exposed to moderate stress conditions, they may initiate a protective response to resist a future severe stress. Thus, they develop dormancy and form storage organs to survive unfavorable environments and to ascertain fast regrowth after that. We investigated the effects of moderate abiotic stresses, namely heat (hot-air and hot-water treatments), cold (0ºC), anaerobiosis, drought and salinity on rhizome growth and outgrowth of lateral rhizomes in *Alstroemeria* cultured *in vitro*. All stresses gave positive results. Under the stress conditions used, mild heat stress gave a high promotion of rhizome growth (maximally *ca.* 75%). Cold stress gave satisfactory results as well. Thus moderate stress increases the sink activity of the rhizome and this likely represents a protective action of the rhizome. These results are consistent with observations that plants grown under stressful conditions tend to allocate a higher proportion of biomass to below-ground biomass (roots and storage organs). In addition, all abiotic stresses increased rhizome multiplication significantly and propagation maximally reached an almost double rate.

We studied apical dominance in *Alstroemeria* cultured *in vitro*. The standard explant was a rhizome (the rhizome tip had not been removed) with two vertically growing shoots from which the larger part had been excised leaving *ca.* 1 cm stem. The outgrowth of the axillary bud located at this 1-cm stem just above the rhizome was examined. Removal of the rhizome tip and the shoot tips released these axillary buds. Replacement of excised tips by an auxin-lanolin mixture restored apical dominance. The auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and *N*-1-napthylphthalamic acid (NPA) reduced apical dominance. The cytokinin 6-benzylaminopurine (BAP) promoted axillary bud

outgrowth. All these results indicate that -even though the architecture of *Alstroemeria* is special- auxin inhibits and cytokinin promotes axillary bud outgrowth just as in other species.

Recently, strigolactones (SLs) have been proposed as branching-inhibiting hormones. They are believed to inhibit bud outgrowth by dampening auxin transport. SL mutants in model plants (*Arabidopsis*, pea, petunia) show an increased branching phenotype and *Arabidopsis* SL mutants have an increased auxin transport capacity. We researched the role of SL in *Alstroemeria*. Explants consisted of a rhizome with either two intact shoots or shoots that had been largely removed. The outgrowth of the axillary bud located at the basal part of the aerial shoot just above the rhizome was observed. Inhibitors of SL biosynthesis (fluridone and D2) increased axillary bud outgrowth. The promotive effect of fluridone was stronger relative to D2. The SL analogue GR24 restored apical dominance. It has been hypothesized that SLs inhibit axillary bud outgrowth by preventing buds to export their auxin into the polar auxin transport stream in the main stem because SLs down-regulate PIN auxin efflux carriers resulting in limited auxin transport capacity. Auxin transport inhibitors (TIBA and NPA) promoted axillary bud outgrowth in the absence fluridone, but were inhibitory in its presence corresponding to the proposed mode of action of SL. Moreover, when SL biosynthesis in the explants was inhibited by adding fluridone, basipetal auxin transport increased strongly in shoot stems and in rhizomes (by *ca*. 350-400%). Furthermore, auxin transport was reduced when GR24 was applied together with fluridone. These results suggest that SLs inhibited axillary bud outgrowth in *Alstroemeria* via dampening of auxin transport.

The results described in this thesis give useful clues for improvement of *Alstroemeria* micropropagation. Even though adaptation of specific nutrient compositions in the culture medium does not bring about significant improvement of rhizome growth, it is worth to adjust experimental procedures in future studies. Applications of moderate abiotic stress to enhance rhizome growth and SL inhibitors such as fluridone to increase rhizome propagation are promising approaches to improve propagation factors, but the after-effects should be evaluated before commercial use is to be embarked upon.

Samenvatting

Alstroemeria is een siergewas waarvan de hybriden worden geteeld voor snijbloem- en potplantproductie. *Alstroemeria* groeit sympodiaal: de rhizoom verandert bij een knoop de groeirichting van horizontaal naar verticaal en genereert een opgaande scheut. De okselknop in deze knoop loopt altijd uit en groeit horizontaal als rhizoom tot de volgende knoop. Daar verandert de rhizoom opnieuw de groeirichting van horizontaal naar verticaal en genereert de volgende opgaande scheut. De okselknop in deze knoop loopt weer uit en continueert de horizontaal groeiende rhizoom. Dit proces herhaalt zich steeds opnieuw. Bij Alstroemeria bestaat een rhizoom bijgevolg uit een 'ketting' van de eerste internodia van uitgelopen, horizontaal groeiende zijscheuten.

De tweede okselknop in de opgaande scheut bevindt zich vlak boven de rhizoom. Gewoonlijk blijft deze knop dormant door sterke apikale dominantie maar soms groeit hij uit tot een zijrhizoom bijv. wanneer de opgaande scheut afsterft of verwijderd is. In de hoger gelegen knopen zijn geen okselknoppen aanwezig. *Astroemeria* heeft in de kas een lage vegetatieve vermeerderingssnelheid door de geringe uitloop van zijrhizomen. Weefselkweekvermeerdering is daarom de aangewezen methode voor commerciële vegetatieve vermeerdering van *Alstroemeria*. In weefselkweek wordt een verhoogde uitloop van zijrhizomen gerealiseerd door toediening van cytokinines aan het medium. Desalniettemin geeft *Alstroemeria* ook in weefselkweek nog steeds een lage vermeerderingsfactor, gewoonlijk beduidend minder dan 2 per cyclus. Dit wordt veroorzaakt door een combinatie van sterke apikale dominantie en slechte rhizoomgroei.

De wetenschappelijk doelstellingen van het onderzoek in dit proefschrift waren inzicht te verkrijgen in consumptie van toegediende nutriënten in relatie tot de groei van rhizomen, het effect van milde abiotische stress op de groei van rhizomen te bepalen en de onderliggende mechanismen van apikale dominantie te onderzoeken. Het praktijkgerichte doel was een snellere vermeerdering van *Alstroemeria* in weefselkweek te verkrijgen door uitlopen en groei van rhizomen te verbeteren.

Slechte rhizoomgroei is één van de belangrijkste hindernissen bij weefselkweekvermeerdering van *Alstroemeria*. Voor snelle vermeerdering moet de zijrhizoom zo snel mogelijk een voldoende grootte bereiken zodat hij van de hoofdrhizoom losgesneden kan worden. Mogelijk zijn bepaalde voedingscomponenten in het medium beperkend en is dat de reden van de trage groe. Daarom werd de consumptie van nutriënten bestudeerd. Consumptie werd vergeleken bij rhizomen die groeiden op 10 en 25 ml standaard MS medium over een periode van 4 weken. Zowel rhizoomgroei als

vermeerdering waren sneller in 25 ml medium. In 10 ml en 25 ml medium werd dezelfde hoeveelheid sucrose opgenomen maar de hoeveelheid anorganische nutriënten die in 25 ml werd opgenomen was tweemaal zo groot als in 10 ml. De waargenomen verschillen tussen de twee volumes kunnen waarschijnlijk worden verklaard door verschillen in diffusiesnelheid en door verschillende dikte van het medium waardoor in 10 ml de diffusieafstanden tot het explantaat groter zijn. NH_4^+ en $H_2PO_4^-$ werden het snelst opgenomen. Mg^{2+} en Ca^{2+} waren na 4 weken kweek nog in hoge concentratie in het medium aanwezig. Uit element-analyse van weefselkweekplantjes bleek dat in rhizomen het niveau van Mg en P vrij laag was, en dat van Ca vrij hoog in vergelijking met de niveaus in opgaande scheuten. Wij veronderstelden daarom dat minder Mg en P, en meer Ca in het weefselkweekmedium de rhizoomgroei zouden kunnen verbeteren. Alleen een reductie van Mg^{2+} naar de helft van de standaardconcentratie verbeterde de rhizoomgroei (met ong. 30%). Bij lagere $H_2PO_4^-$ en hogere Ca^{2+} concentratie, was de rhizoomgroei niet beter.

Wanneer planten aan milde stress worden blootgesteld, starten ze vaak acties om zichzelf te beschermen. Dat maakt ze ook resistent tegen zware stress en biedt bescherming op de korte termijn. Er is ook bescherming op de lange termijn. Om zich te beschermen tegen ongunstige klimaatomstandigheden, ontwikkelen planten vaak speciale overlevingsorganen die in rust gaan vóór de ongunstige omstandigheden en reserves bevatten voor snelle hergroei wanneer de ongunstige periode is afgelopen. Wij onderzochten de effecten van milde abiotische stress namelijk hoge temperatuur (warme-lucht en warm-water behandelingen), koude (0ºC), anaerobiosis, droogte en een hoog zoutgehalte op rhizoomgroei en uitloop van zijrhizomen in *Alstroemeria*. Alle stressen gaven een positief resultaat. Bij de geteste condities gaf bijv. milde wamte een bevordering van de rhizoomgroei (maximaal ong. 75%). De groei van de opgaande scheuten werd geremd of was onveranderd. De milde stress verhoogde dus de *sink*-activiteit van de rhizoom. Dit is waarschijnlijk onderdeel van een beschermende reactie. Dit resultaat komt overeen met waarnemingen door ecologen dat planten bij zware negatieve omstandigheden er naar neigen om een groter deel van de biomassa onder de grond te alloceren (in wortels en opslagorganen). Alle abiotische stressen verhoogden bovendien de rhizoomuitloop significant en de vermeerdering werd bijna verdubbeld.

Wij bestudeerden eveneens apikale dominantie. Het standaard explantaat was een rhizoom met twee opgaande scheuten waarvan het grootste deel was weggesneden en er nog ong. 1-cm scheut over was; de rhizoomtop werd niet verwijderd. We onderzochten de

uitgroei van de okselknop die op deze 1-cm scheut vlak boven de rhizoom aanwezig was. Verwijdering van de rhizoomtop en/of de scheuttop stimuleerde de uitgroei van deze okselknop. Wanneer de top werd afgesneden en vervangen door een auxine-lanoline pasta werd de apikale dominantie hersteld. De auxinetransport inhibitoren 2,3,5-triiodobenzoic acid (TIBA) en *N*-1-naphthylphthalamic zuur (NPA) verminderden apikale dominantie. Het cytokinine 6-benzylamine (BA) bevorderde okselknopuitgroei. Deze resultaten wijzen erop dat -hoewel de architectuur van *Alstroemeria* heel verschillend is van die van modelgewassen- het mechanisme van apikale dominantie identiek is: auxine afkomstig uit de apex remt en cytokinine bevordert okselknopuitloop.

Recent zijn strigolactonen (SLs) ontdekt als hormonen die de uitloop van okselknoppen remmen. Men veronderstelt dat zij knopuitloop remmen door auxinetransport te reduceren. SL mutanten in modelplanten (*Arabidopsis*, erwt, petunia) vertonen een fenotype met verhoogde vertakking en SL mutanten van *Arabidopsis* hebben een hogere auxinetransport capaciteit. Wij onderzochten de rol van SL in *Alstroemeria*. Explantaten bestonden uit een rhizoom met twee intacte scheuten of scheuten die grotendeels waren verwijderd. We scoorden de uitgroei van de okselknop die gelokaliseerd is op de opgaande scheut net boven de rhizoom. Remmers van de biosynthese van SL (fluridon en D2) verhoogden okselknopuitloop. De bevordering door fluridon was het grootst. De SL analoog GR24 herstelde apikale dominantie. In de literatuur (O. Leyser) heeft men de hypothese opgesteld dat okselknopuitgroei geremd worden door SLs doordat okselknoppen verhinderd worden om hun auxine in de polaire auxine-stroom van de hoofdstengel te exporteren. SLs blokkeren nl. PIN synthese en daarmee het polaire auxinetransport. De auxinetransport remmers TIBA en NPA bevorderden okselknopuitgroei in afwezigheid van fluridon, maar waren remmend als fluridon aanwezig was. Dit komt overeen met de wijze van actie van SL voorgesteld door Leyser. Wanneer de biosynthese van SL door fluridon werd geremd, steeg het basipetale auxinetransport sterk in zowel de opgaande scheuten als in de rhizoom (met ong. 350-400%). Het auxinetransport werd verminderd wanneer GR24 samen met fluridon werd toegediend. Deze resultaten wijzen er op dat SLs okselknopuitgroei in *Alstroemeria* remmen via het reduceren van auxinetransport.

De resultaten die in deze thesis worden beschreven geven verschillende aanwijzingen voor verbetering van weefselkeekvermeerdering van *Alstroemeria*. Alhoewel de aanpassing van voeding geen significante verbetering van de rhizoomgroei bewerkstelligde, is het de moeite waard om voeding in weefselkweek verder te bestuderen. Toepassing van milde abiotische stress en van SL remmers zoals fluridon, is veelbelovend

om de rhizoomgroei en/of uitloop te verbeteren. Eventuele nawerking moet worden geëvalueerd voor commerciële toepassing.

สรุป

อัลสโตรมีเลียเป็นไม้ดอกไม้ประดับที่มีมูลค่าทางเศรษฐกิจสูง มีการผลิตพันธุ์ลูกผสมเพื่อใช้เป็นไม้ตัดดอกและไม้กระถางเป็นหลัก พืชชนิดนี้มีการเจริญเติบโตไปทางแนวนอน หรือที่เรียกกว่า sympodial มีลักษณะการเจริญเติบโตที่เฉพาะ คือ ที่ข้อปลายสุดของเหง้า (rhizome) มีปลายยอดเหง้า (rhizome apex) ซึ่งจะเปลี่ยนทิศทางการเจริญเติบโตจากแนวนอนไปเป็นแนวตั้ง จากนั้นจึงพัฒนาเป็นต้นเหนือดิน (aerial shoot) แท้จริงแล้วปลายยอดเหง้าก็คือ ตาข้างอันดับแรกของต้นเหนือดิน ตาข้างอันนี้จะแตกออกมาในแนวนอนเสมอ เป็นปลายยอดเหง้าอันใหม่ และเกิดการสร้างปล้องเหง้าอันใหม่ ต่อจากนั้นที่ข้อเหง้าอันถัดไป ปลายยอดเหง้าก็จะเปลี่ยนทิศทางการเจริญเติบโตจากแนวนอนไปเป็นแนวตั้งและสร้างต้นเหนือดินต้นใหม่อีกครั้งเช่นเดิม การเจริญเติบโตของตาข้างอันดับแรกมีรูปแบบซ้ำๆ เช่นนี้ ซึ่งจะเกิดการสร้างปล้องของเหง้า ดังนั้นเหง้าจะประกอบด้วยฐานปล้องยาวต่อเนื่องกัน ซึ่งเกิดพร้อมๆ ไปกับการสร้างต้นเหนือดิน

ส่วนตาข้างอันดับที่สองของต้นเหนือดินจะอยู่ถัดไปด้านบนของตาข้างอันดับแรก ตาข้างอันนี้มักจะอยู่ในระยะพักตัวเนื่องจากอัลสโตรมีเลียมีสภาวะตายอดข่มตาข้าง (apical dominance) ในระดับสูง แต่ตาข้างอันดับที่สองนี้มีการแตกยอดและพัฒนาไปเป็นเหง้าด้านข้าง (lateral rhizome) ได้ในบางสภาวะ เช่น เมื่อต้นเหนือดินตายหรือถูกตัดออกไป นอกจากนี้ไม่พบว่ามีการพัฒนาของตาข้างที่ซอกใบของต้นเหนือดิน ด้วยเหตุนี้จึงทำให้อัลโตรมีเลียเป็นพืชที่มีอัตราการขยายพันธุ์ต่ำตามธรรมชาติ เนื่องจากการพัฒนาของเนื้อเยื่อเจริญของตาข้างที่จำกัด การขยายพันธุ์พืชด้วยการเพาะเลี้ยงเนื้อเยื่อ (micropropagation) เป็นวิธีที่นิยมใช้ในการผลิตอัลโตรมีเลียในระดับการค้า มีวิธีการ คือ กระตุ้นให้สร้างเหง้าด้านข้างจากการบังคับให้เกิดการแตกตาข้างอันดับที่สองของต้นเหนือดินที่กำลังเจริญเติบโต อย่างไรก็ตาม วิธีการนี้ยังให้อัตราการขยายพันธุ์ที่ต่ำ (น้อยกว่า 2 ต่อรอบการเพาะเลี้ยง) ทั้งนี้มีสาเหตุร่วมกันระหว่างลักษณะตายอดข่มตาข้างที่สูงและการเจริญเติบโตของเหง้าที่ต่ำ

วิทยานิพนธ์นี้มีวัตถุประสงค์โดยทั่วไป คือ เพื่อปรับปรุงการขยายพันธุ์อัลสโตรมีเลียด้วยการเพาะเลี้ยงเนื้อเยื่อ โดยเพิ่มการเจริญเติบโตและเพิ่มปริมาณของเหง้า นอกจากนี้มีวัตถุประสงค์ในเชิงวิทยาศาสตร์ ซึ่งเน้นการศึกษาระดับพื้นฐานและประยุกต์ในหัวข้อต่อไปนี้ (1) เพื่อศึกษาความสัมพันธ์ของการนำสารอาหารไปใช้ระหว่างรอบของการขยายพันธุ์และการเจริญเติบโตของเหง้า (2) เพื่อศึกษาอิทธิพลและผลกระทบของความเครียดจากสิ่งไม่มีชีวิตในระดับปานกลาง (moderate abiotic stress) ต่อการเจริญเติบโตของเหง้า และ (3) เพื่อศึกษากลไกตายอดข่มตาข้างในการควบคุมการแตกของตาข้าง

การเจริญเติบโตที่ต่ำของเหง้าเป็นอุปสรรคสำคัญของการขยายพันธุ์อัลสโตรมีเลียด้วยการเพาะเลี้ยงเนื้อเยื่อ ในการขยายพันธุ์นั้นเหง้าด้านข้างควรมีขนาดใหญ่เพียงพอ เพื่อที่จะสามารถแยกออกมาจากเหง้าหลักได้เร็วที่สุดเท่าที่จะเป็นไปได้ ทั้งนี้ได้ศึกษาการนำสารอาหารไปใช้ระหว่างรอบการเพาะเลี้ยง

เนื้อเยื่อเพื่อที่จะปรับปรุงการเจริญเติบโตของเหง้า โดยเปรียบเทียบระหว่างอาหารแข็งสูตร MS ปริมาตร 10 และ 25 มล. ในระยะเวลา 4 สัปดาห์ พบว่า เหง้ามีการเจริญเติบโตและเพิ่มปริมาณสูงกว่าในอาหาร 25 มล. ในอาหารทั้ง 10 และ 25 มล. มีการนำน้ำตาลซูโครสไปใช้ในปริมาณที่เท่าๆ กัน แต่การนำสารอาหารอนินทรีย์ไปใช้ในอาหาร 25 มล. มีปริมาณสูงกว่าอาหาร 10 มล. ประมาณสองเท่า ความแตกต่างที่เกิดขึ้นในอาหารที่มีปริมาตรแตกต่างกันนี้ อาจจะอธิบายได้จากอัตราการแพร่ที่แตกต่างกันของโมเลกุลและความหนาของอาหารแข็ง ซึ่งในอาหาร 10 มล. มีระยะทางการแพร่ถึงชิ้นส่วนพืช (explants) ที่กว้างกว่า นอกจากนี้พบว่า NH_4^+ และ $H_2PO_4^-$ ถูกนำไปใช้อย่างรวดเร็ว ในขณะที่ Mg^{2+} และ Ca^{2+} ยังเหลือความเข้มข้นค่อนข้างสูง หลังจาก 4 สัปดาห์ การวิเคราะห์องค์ประกอบของธาตุในพืชที่ได้จากการเพาะเลี้ยงเนื้อเยื่อ พบว่า ในเหง้ามีระดับ Mg และ P ค่อนข้างต่ำ และ Ca ค่อนข้างสูงเมื่อเปรียบเทียบกับลำต้นและใบ จึงได้ตั้งสมมติฐานว่าความเข้มข้นที่ลดลงของ Mg และ P และความเข้มข้นที่เพิ่มขึ้นของ Ca ในอาหารอาจจะช่วยปรับปรุงการเจริญเติบโตของเหง้า ปรากฏว่า มีเพียงการลด Mg^{2+} ลงครึ่งหนึ่งของระดับปกติตามสูตรมาตรฐาน MS เท่านั้นที่ปรับปรุงการเจริญเติบโตของเหง้า (ประมาณ 30%) แต่การลดความเข้มข้นของ $H_2PO_4^-$ หรือเพิ่มความเข้มข้นของ Ca^{2+} ไม่สามารถปรับปรุงการเจริญเติบโตของเหง้าเมื่อเปรียบเทียบกับทรีทเมนต์ควบคุม สันนิษฐานว่าการเพิ่มการเจริญเติบโตของเหง้านั้นไม่น่าจะมีอิทธิพลมาจากสารอาหารที่เฉพาะ

เมื่อพืชได้รับสภาวะเครียดระดับในปานกลาง พืชอาจจะสร้างกลไกปกป้องตัวเองเพื่อให้ต้านทานต่อสภาวะเครียดที่รุนแรงกว่าในอนาคต เช่น การเข้าสู่ระยะพักตัว (dormancy) และการสร้างอวัยวะสะสมอาหาร (storage organ) เพื่อให้อยู่รอดภายใต้สภาพแวดล้อมที่ไม่พึงประสงค์ได้ และเพื่อให้มีการเจริญเติบโตครั้งใหม่ได้รวดเร็วหลังจากนั้น ทั้งนี้ได้ศึกษาผลของความเครียดจากสิ่งไม่มีชีวิตในระดับปานกลาง ได้แก่ ความร้อน (อากาศร้อนและน้ำร้อน) ความเย็น (0 องศาเซลเซียส) การขาดอากาศ (anaerobiosis) ความแห้งแล้ง และความเค็ม ต่อการเจริญเติบโตของเหง้าและการสร้างเหง้าด้านข้างในอัลโตรมีเลียในสภาพเพาะเลี้ยงเนื้อเยื่อ พบว่า ความเครียดทุกชนิดให้ผลในเชิงบวก ความเครียดที่เกิดจากการให้ความร้อนในระดับปานกลางนั้นกระตุ้นการเจริญเติบโตของเหง้าได้สูง (สูงสุดประมาณ 75%) ส่วนความเย็นให้ผลเป็นที่น่าพอใจเช่นกัน ดังนั้นความเครียดในระดับปานกลางเพิ่มความสามารถในการสะสม (sink activity) ของเหง้า ซึ่งเป็นไปได้ว่าสิ่งนี้เป็นกลไกการป้องกันตัวของเหง้า ผลการทดลองเหล่านี้สอดคล้องกับการศึกษาในพืชอื่นๆ ที่เจริญภายใต้สภาวะเครียด ซึ่งมีแนวโน้มว่าจะมีการเคลื่อนย้ายชีวมวล (biomass) ส่วนใหญ่ไปยังส่วนที่อยู่ใต้ดิน (รากและอวัยวะสะสมอาหาร) นอกจากนี้พบว่า ความเครียดจากทุกประเภทยังเพิ่มปริมาณของเหง้าอย่างมีนัยสำคัญ และเพิ่มการขยายพันธุ์สูงสุดเกือบสองเท่า

ในการศึกษากลไกตายอดข่มตาข้างของอัลสโตรมีเลียในสภาพเพาะเลี้ยงเนื้อเยื่อ ได้ใช้ชิ้นส่วนพืชมาตรฐาน คือ ส่วนของเหง้า (ปลายยอดเหง้าไม่ได้ถูกตัดออก) ที่มีต้นเหนือดินสองต้น ซึ่งถูกตัดส่วนปลายออกคงเหลือส่วนลำต้นไว้ประมาณ 1 ซม. โดยได้ตรวจสอบการแตกตาข้างอันดับที่สองของต้นเหนือ

ดิน ปรากฏผลดังนี้ การตัดปลายยอดของเหง้าและต้นเหนือดินทิ้งทำให้เกิดการแตกตาข้าง การแทนที่ปลายยอดที่ถูกตัดออกด้วยส่วนผสมของออกซิน-ลาโนลิน (auxin-lanolin mixture) ทำให้สภาวะตายอดข่มตาข้างกลับคืนมา สารยับยั้งการขนส่งออกซิน (auxin transport inhibitors) ได้แก่ 2,3,5-triiodobenzoic acid (TIBA) และ *N*-1-napthylphthalamic acid (NPA) ลดสภาวะตายอดข่มตาข้าง ไซโตไคนิน (cytokinin) ได้แก่ 6-benzylaminopurine (BAP) กระตุ้นการแตกตาข้าง ผลการศึกษาทั้งหมดนี้บ่งชี้ว่าถึงแม้อัลสโตรมีเลียจะมีลักษณะโครงสร้างที่พิเศษ แต่ก็ถูกออกซินยับยั้งและไซโตไคนินกระตุ้นการแตกตาข้างได้เช่นเดียวกันกับพืชสปีชีส์อื่นๆ

ในปัจจุบันมีการค้นพบฮอร์โมนชนิดใหม่ที่ยับยั้งการแตกกิ่งก้านของพืชมีชื่อว่า strigolactone (SL) เชื่อกันว่าฮอร์โมนชนิดนี้ยับยั้งการแตกของตาโดยลดการขนส่งออกซิน มีการพบว่า ในพืชต้นแบบ (*Arabidopsis*, ถั่วลันเตา, พิทูเนีย) สายพันธุ์กลายของยีน SL มีลักษณะแตกกิ่งก้านที่เพิ่มขึ้น และใน *Arabidopsis* สายพันธุ์กลายของยีน SL มีการขนส่งออกซินเพิ่มขึ้น ในวิทยานิพนธ์นี้ได้ทำการศึกษาบทบาทของ SL ในอัลโตรมีเลีย ชิ้นส่วนพืชที่ใช้ประกอบด้วยเหง้าที่มีต้นเหนือดินทั้งต้นหรือส่วนใหญ่ของต้นถูกตัดออก และได้ตรวจสอบการแตกของตาข้างอันดับที่สองที่อยู่ส่วนฐานของต้นเหนือดินซึ่งอยู่เหนือเหง้าขึ้นไปด้านบน พบว่า สารยับยั้งการสังเคราะห์ SL ได้แก่ fluridone และ D2 เพิ่มการแตกตาข้าง โดย fluridone มีผลกระตุ้นสูงกว่า D2 ขณะที่สารคล้าย SL ได้แก่ GR24 มีผลทำให้เกิดสภาวะตายอดข่มตาข้าง มีการตั้งสมมติฐานว่า SL ยับยั้งการแตกตาข้างโดยป้องกันการขนส่งออกซินจากตาเข้าสู่กระแสการขนส่งออกซินในลำต้น เพราะว่า SL ลดการสังเคราะห์โปรตีน PIN ที่มีหน้าที่กำหนดการขนส่งออกซินอย่างมีทิศทาง ส่งผลให้การขนส่งออกซินถูกจำกัด สารยับยั้งการขนส่งออกซิน (TIBA และ NPA) กระตุ้นการแตกตาข้าง แต่กลับมีผลยับยั้งเมื่อมี fluridone ร่วมอยู่ด้วย ซึ่งสอดคล้องกับกลไกการออกฤทธิ์ของ SL ที่ถูกเสนอขึ้นมา นอกจากนี้การสังเคราะห์ SL ในชิ้นส่วนพืชถูกยับยั้งการเมื่อมีการเติม fluridone ซึ่งพบว่า การขนส่งออกซินจากยอดไปสู่ส่วนฐาน (basipetal auxin transport) เพิ่มขึ้นอย่างมากทั้งในลำต้นของต้นเหนือดินและเหง้า (ประมาณ 350-400%) ในขณะที่การขนส่งออกซินลดลงเมื่อมีการเติม GR24 ร่วมกับ fluridone ผลที่ได้นี้ชี้แนะว่า SL ยับยั้งการแตกตาข้างในอัลสโตรมีเลียโดยลดการขนส่งออกซิน

ผลการศึกษาที่อธิบายไว้ในวิทยานิพนธ์นี้ ให้แนวทางที่มีประโยชน์ต่อการปรับปรุงการขยายพันธุ์อัลสโตรมีเลียด้วยการเพาะเลี้ยงเนื้อเยื่อ แม้ว่าการปรับองค์ประกอบสารอาหารที่เฉพาะในอาหารเพาะเลี้ยงไม่ได้ให้ผลการปรับปรุงที่มีนัยสำคัญ จึงสมควรจะปรับกระบวนการทดลองในการศึกษาครั้งต่อไป การใช้ความเครียดจากสิ่งไม่มีชีวิตระดับในปานกลางเพื่อส่งเสริมการเจริญเติบโตของเหง้า และการใช้สารยับยั้งการสังเคราะห์ SL อย่าง fluridone เพื่อเพิ่มปริมาณเหง้านั้นเป็นวิธีที่น่าสนใจสำหรับใช้ในปรับปรุงอัตราการขยายพันธุ์ อย่างไรก็ตามควรมีการประเมินผลกระทบก่อนที่จะตัดสินใจนำไปใช้ในเชิงการค้า

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PAWEENA PUMISUTAPON

Wageningen, the Netherlands
March, 2012

About the author

Paweena Pumisutapon was born on June 15th 1974 in Chiangmai, Thailand. She received her BSc in Agriculture in 1995 at Chaingmai University and MSc in Biotechnology in 1999 at Chulalongkorn University. She was awarded the scholarship for her MSc studies from the Office of the Higher Education Commission of Thailand. Since 1999 to present, she works as a lecturer for the Biotechnology Program at Maejo University, Chiangmai, Thailand. In 2007, she was awarded the scholarship from the Ministry of Science and Technology of Thailand to pursue her PhD studies at Wageningen UR - Plant Breeding. This thesis presents the outcome of her PhD research from 2007 to 2012.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Paweena Pumisutapon
Date: 8 May 2012
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	-
Apical Dominance and Growth In Vitro of *Alstroemeria*	17 December 2007
► **Writing or rewriting a project proposal**	
Apical Dominance and Growth In Vitro of *Alstroemeria*	14 January 2008
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Radiation Course-5B 'Safe Handling of Radioactive Materials and Sources'	4-6 November 2008
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Days, Wageningen	13 September 2007
EPS PhD Student Days, Leiden	26 February 2009
► **EPS theme symposia**	
ESP Theme 3 Symposium: 'Metabolism and Adaptation', Wageningen	6 November 2007
ESP Theme 4 Symposium: 'Genome Plasticity', Wageningen	12 December 2008
ESP Theme 4 Symposium: 'Genome Plasticity'	14 December 2009
► **NWO Lunteren days and other National Platforms**	
NVPW Autumn Symposium, Leiden	2 November 2007
NVPW Spring Symposium, Wageningen	14 March 2008
NVPW Autumn Symposium, Leiden	24 October 2008
NVPW Spring Symposium, Wageningen	20 March 2009
NVPW Autumn Symposium, Wageningen	20 November 2009
NVPW Autumn Symposium, Wageningen	26 November 2010
► **Seminars (series), workshops and symposia**	
EPS flying seminar: Prof. Scott Phoetig	24 September 2007
EPS flying seminar: Prof. Hiroo Fukuda	26 November 2007
EPS flying seminar: Prof. Simon Gilroy	19 May 2008
Seminar: Prof. Harro Bouwmeester	30 November 2007
Seminar: Dr. Joost Lucker	18 December 2007
Seminar: Prof. Jaakkko Kangasjarvi	13 March 2008
Plant Sciences Seminar: Prof. Ton Bisseling and Prof. Harro Bouwmeester	8 September 2009
Plant Sciences Seminar: Prof. Olaf van Kooten and Prof. Jack Leunissen	13 October 2009
Plant Sciences Seminar: Prof. Ken Giller and Prof. Richard Visser	8 December 2009
Plant Sciences Seminar: Prof. Marcel Diceke and Prof. Marcel Janson	11 January 2010
Plant Sciences Seminar: Prof. Martin van Ittersum, Jan Verhagen and Dr. Gerard van der Linden	8 February 2011
EPS Symposium "Ecology and Experimental Plant Sciences 2"	22 September 2009
Plant Breeding Research Day 2007, Wageningen	27 September 2007
Plant Breeding Research Day 2008, Wageningen	17 June 2008
Plant Breeding Research Day 2009, Wageningen	3 March 2009
Plant Breeding Research Day 2010, Wageningen	8 February 2010
► **Seminar plus**	
► **International symposia and congresses**	
6th International Symposium on IVCHB, Brisbane	24-28 August 2008
12th International Association for Plant Biotechnology, Missouri	6-11 June 2010
► **Presentations**	
Poster presentation: Plant Breeding Research Day 2008, Wageningen	17 June 2008
Oral presentation: 6th International Symposium on IVCHB, Brisbane	24-28 August 2008
Oral presentation: NVPW Autumn Symposium, Leiden	24 October 2008
Oral presentation: Plant Physiology and Genetics (PPH + GEN) Colloquia, Wageningen	23 March 2009
Poster presentation: 12th International Association for Plant Biotechnology, Missouri (2 posters)	6-11 June 2010
► **IAB interview**	12 January 2010
► **Excursions**	
Van Zanten Plants, Rijsenhout and Könst Alstroemeria, Nieuwveen	13 June 2007
Van Zanten Plants, Rijsenhout and Könst Alstroemeria, Nieuwveen	20 August 2007
Subtotal Scientific Exposure	*17.0 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
5th Utrect PhD Summerschool on Environmental Signaling, Utrecth	24-26 August 2009
Confocal Light Microscopy	25-28 May 2010
► **Journal club**	
Member of a journal discussion group at Plant Breeding	2007-2010
► **Individual research training**	
Subtotal In-Depth Studies	*5.4 credits**

4) Personal development	date
► **Skill training courses**	
PhD Information Literacy, including introduction Endnote	11-12 September 2007
Endnote 9 advanced	4 October 2007
Searching for Science on the Web	10 October 2007
Techniques for Writing and Presenting a Scientific paper	16-19 October 2007
PhD Competence Assessment	13 November 2007
Scientific Publishing	29 November 2007
Advance Course Guide to Scientific Artwork	15-16 December 2008
PhD Course Basic Statistics	29-30 June and 1-3 July 2009
Introduction to R course	8-9 October 2009
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*5.3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**36.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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