Development of two *in vitro* regeneration systems through leaf explant and callus culture and the application for genetic transformation in *Alstroemeria*

De ontwikkeling van twee "*in vitro* vermeerderingssystemen", één via bladexplantaten en de ander via callus cultuur en de toepassing van deze vermeerderingssystemen voor genetische transformatie in *Alstroemeria*

建立百合水仙葉培植體及癒傷組織之微體繁殖體系以及其應用於基因轉殖之研究

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Bibliographic Abstract: This thesis describes the establishment of two plant regeneration systems in *Alstroemeria*. The first one used leaf explants as starting material, and the second one used stem segments. Leaf explants were induced to form adventitious shoots via the organogenesis pathway, which developed into plants. The stem segments were induced to form calli, which developed into plants via the embryogenesis pathway. Both systems were developed for plant propagation as well as for genetic transformation purpose. Leaf-explant culture method is an alternative way of micropropagation, that can enhance the propagation efficiency. Callus culture system was successfully used in particle gun-mediated transformation, and many transgenic plants were obtained. Two plasmids were investigated, one containing a firefly luciferase gene, and another one containing a phosphinotricin acetyl transferase gene (PAT, a herbicide resistance gene) together with a ß-glucuronidase gene (GUS). Selection based on either the luciferase activity, or phosphinotricin resistance proved to be effective. Luciferase gene, PAT gene, and GUS gene were transformed into the *Alstroemeria* plants with high efficiency.

Keywords: *Alstroemeria*, Alstroemeriaceae, embryogenesis, callus culture, friable, granular, GUS, leaf explant, luciferase, organogenesis, particle bombardment, phosphinotricin, regeneration, transformation

謹以此書 獻給
月夏、我們敬愛的父親與母親
以及宛青、鈺臻

Contents

Propositions

1. The leaf-explant based regeneration system is not appropriate for the development of genetic transformation with particle gun (this thesis).

2. Combination of the leaf explant regeneration system with that of the rhizome multiplication system, is enhancing the multiplication efficiency of *Alstroemeria* (this thesis).

3. In *Alstroemeria* the percentage of responding leaf explants with rhizome formation is more important than the amount of shoots regenerated per leaf explant, because rhizome formation is the most important factor for micropropagation (this thesis).

4. A plant regeneration system, based on granular callus with dividing cells near the surface, is a prerequisite for genetic transformation (this thesis).

5. This thesis contains basic information for successful transformation of existing varieties of *Alstroemeria.*

6. Successful plant breeding is always a mixture of art and science, and invariably based on genetic principles.

7. Things are going well when they are managed leisurely (Chinese proverb).

8. Inadequate knowledge of the Dutch language has the advantage, in that it prevents you from being influenced by all kinds of public media, and lets you concentrate your mind on your own job. The disadvantage is, you never exactly know what your colleagues were talking about in the canteen.

9. The public debate about food products obtained from genetic engineering will also be an issue in Taiwan.

10. Scientists are always forced to face the 'impossible' challenge.

11. A ripe rice-plant has a bent neck (Taiwanese proverb).

These propositions are attached to the thesis "Development of two in vitro regeneration systems through leaf explant and callus culture and the application for genetic transformation in *Alstroemeria*" by Hsueh-Shih Lin, for the public defence held on Wednesday, September 9, 1998, in Wageningen.

Chapter 1

General introduction

The *Alstroemeria* plant

Alstroemeria, also known as Inca lily, Peruvian lily, or even called 'Narcissus lily' in Taiwan, is a South America endemic monocotyledonous plant species mainly found in Chile and Brazil (Aker and Healy 1990). It belongs to the family Alstroemeriaceae according to Dahlgren and Clifford (1982) and is commonly called by its genus name. Over the last two decades *Alstroemeria* has become a popular greenhouse-grown cut flower in The Netherlands, due to its low-energy requirement, long vase-life, and wide range of flower colors.

An *Alstroemeria* plant consists of fleshy rhizomes, aerial shoots, and thick tuberous roots. Rhizome is an underground-grown organ with many stem-like nodes, and at each node one aerial shoot and some roots originate. The rhizome has a sympodial growth habit (Bayer 1987; Buxbaum 1951). Morphological research demonstrated that the rhizome is actually the first axillary shoot of the aerial sprout (Buxbaum 1951). The aerial shoot is either a vegetative stem with leaves only or a generative stem with flowers in its apex. The leaf usually rotates 180° at its base, so that the adaxial surface faces down.

Chromosome studies reveal that the *Alstroemeria* species are mostly diploid with 16 chromosomes (2n=2x=16), and the commercial cultivars are not only diploid, but also triploid (2n=3x=24), tetraploid (2n=2x=32), or even aneuploid (with chromosome number of 25, 31, 33 etc.; Hang and Tsuchyia 1988; Tsuchyia et al. 1987; Tsuchyia and Hang 1987,1989). The spontaneous polyploidy induction observed in breeding cultivars is probably due to the production of unreduced gametes during meiosis (Ramanna 1991).

Plant regeneration and *in vitro* propagation of *Alstroemeria*

Conventionally, *Alstroemeria* is propagated by rhizome division, but the multiplication rate is rather low, so that tissue culture techniques were developed (Bond and Alderson 1993; Pierik et al. 1988; Van Zaayen et al. 1992; Ziv et al. 1973). However, the multiplication efficiency is still rather low, especially for the so called 'Butterfly type' cultivars. Therefore, *Alstroemeria* was classified as an *in vitro* recalcitrant plant species due to its low multiplication rate and its limited number of regenerable tissues. An overview of the successful plant regeneration events reveals that the initial plant material used in *Alstroemeria* is limited to the rhizome, and to the zygotic

embryo/ovule (Table 1). The other plant organs tested rarely gave a response or no response at all. Since true-to-type is the most important requirement for commercial plant propagation, the rhizome is still the major propagation unit. In addition, the development of alternative *in vitro* multiplication systems based on other tissues is desirable.

The ovule culture system was developed for plant breeding purpose in order to overcome interspecific crossing barriers. The callus culture system, that was initiated from zygotic embryos, is not useful for multiplication of the existing cultivars. Moreover, the potential somaclonal variation should be taken into account too. Therefore those two methods, based on zygotic embryos, are not further useful for micropropagation purpose. However, the callus culture system may play an important role in genetic modification.

Table 1. Successful *in vitro* plant regeneration systems in *Alstroemeria*.

Initial material	Type of regeneration	Reference
Rhizome meristem	Organogenesis	Hakkaart and Versluijs, 1988 Van Zaayen et al., 1992
Rhizome segment	Organogenesis	Gabryszewska, 1995 Gabryszewska and Hempel, 1985 Bond and Alderson, 1993
Rhizome tip	Organogenesis	Bond and Alderson, 1993 Buitendijk, 1998 Lin and Monette, 1987 Pierik et al., 1988
Zygotic embryo	Embryogenesis	Gonzalez-Benito and Alderson, 1992 Hutchinson et al., 1994 Hutchinson et al., 1997 Van Schaik et al., 1996
Zygotic ovule	Embryogenesis	Buitendijk et al., 1995 De Jeu and Jacobsen, 1995 Lu and Bridgen, 1996 Ishikawa et al., 1997

Genetic transformation in monocots

Traditional breeding objectives of *Alstroemeria* were mainly focused on the improvement of horticultural important characteristics. The mutation breeding techniques, successfully

developed in 1970-1980 (Broertjes and Van Harten 1988), gave a tremendous contribution. Moreover, embryo rescue techniques developed in recent years, which have overcome the interspecific crossing barriers (Buitendijk et al. 1995; De Jeu and Jacobsen 1995), are useful for the introduction of genes from wild species into cultivars. However, some diseases and post-harvest problems could not easily be solved with these tools, because the resistance or tolerance factors involved were not localized yet in the wild species. Gene transformation, mediated by the *Agrobacterium* vector system or particle delivery systems developed in recent years, is considered to be another promising tool for plant improvement. By these techniques the resistance/tolerance genes from other plant species or even from animals could be introduced into the target plants without crossing.

To accomplish successfully gene transformation, four important parameters are required: a) an efficient DNA delivery system, b) appropriate target cells competent for transformation and regeneration, c) adequate promoter systems for stable gene expression, and d) a good selection system (Christou 1995, 1997; Hiei et al. 1997. Jähne et al. 1995; Smith and Hood 1995).

a. an efficient DNA delivery system

Although monocotyledonous plants were not standing outside the host range of *Agrobacterium tumefaciens*, the transformation efficiency mediated by this vector was still low and many parameters should be improved before it becomes a routine used technology in monocots (Hiei et al. 1997; Smith and Hood 1995). On the other hand, particle bombardment has become a popular choice in monocots, because there is no host range limitation with this method and the transformation efficiency is relatively high in combination with an efficient selection method (Christou 1995, 1997; Jähne et al. 1995). Many successful results reported in recent years in monocots are listed in Table 2.

b. The appropriate target cells competent for transformation and regeneration

The development of a system that may provide appropriate target cells with competence for both transformation and regeneration is a prerequisite for transformation (Christou 1995;

Raemakers et al. 1997; Taylor et al 1996). Compared to dicotyledonous plants, the kind of tissue capable of regeneration is rarely found in monocots. Therefore, tissues of zygotic embryos and their derivative callus are usually used (Table 2).

Table 2. Successful particle bombardment-mediated gene transformations in important monocotyledonous crops.

Species	Target tissue	Reference
Avena	Callus, from embryo	Torbert et al., 1998
Asparagus	Suspension callus Callus, from seed	Li and Wolyn, 1997 Cabrera-Ponce et al., 1997
Gladiolus	Callus, from corm	Kamo et al., 1995
Hordeum	Microspore Embryo scutellum	Jähne et al., 1994 Koprek et al., 1996
Lilium	Callus, from bulblet scale	Watad et al., 1998
Oryza	Callus, from embryo	Abedinia et al. 1997
Saccharum	Callus, from immature inflorescence	Gallo-Meagher and Irvine, 1996
Sorghum	Inflorescence	Casas et al. 1997
Triticum	Embryo scutellum Embryo	Takumi and Shimada, 1997 Wang et al., 1996
Zea	Embryo scutellum Embryo	Brettschneider et al., 1997 Kemper et al., 1996

c. an adequate promoter for gene expression

Gene expression efficiency is closely related to the promoter. Nowadays, many promoters are found to be useful for transformation of monocots, including *Act1* (actin)(McElroy et al. 1991; Zhang et al. 1991) from rice, *Adh1* (alcohol dehydrogenase) (Dennis et al. 1984) from maize, CaMV 35S from cauliflower mosaic virus (Odell et al. 1985), and *Ubi1* (ubiquitin) (Christensen et al. 1992) from maize. The widely used CaMV 35S promoter has proved to be effective in dicots, as well as in the monocot gladiolus (Kamo et al. 1995) and onion (Eady et al. 1996). It was less effective in cereal/grass (Taylor et al. 1993), maize (Christensen et al. 1992), oil palm (Chowdhury et al. 1997), rice (Li et al. 1997), and

wheat (Ortiz et al. 1997). All of the above mentioned promoters gave rise to transformants in different monocots suggesting that the appropriate promoter has to coordinate with the appropriate plant species.

d. a good selection system

Wilmink and Dons (1993) concluded that the choice of a good selection system is also an important factor for transformation, because even the most successful transformation systems for monocotyledonous plants showed a low efficiency. The selection system is mainly based on two groups of agent: antibiotics and herbicides. The widely used antibiotic 'kanamycin' was considered to be less effective in monocots, because cells and tissues of monocots are relatively insensitive to this antibiotic (Wilmink and Dons 1993). However, some successful events did not agree with that, for example in asparagus (Li and Wolyn, 1997) and in *Dendrobium* (Kuehnle and Sugii 1992). Another commonly used antibiotic is 'hygromycin' successful in transformation of creeping bentgrass (Xiao and Ha 1997) and in rice (Abedinia et al. 1997).

The herbicide phosphinotricin (PPT), and the tri-peptide PPT compound 'bialaphos' are widely used as selection agent in the transformation of monocots. PPT was successfully used in barley (Jähne et al. 1994), gladiolus (Kamo et al. 1995), lily (Watad et al. 1998), and orchardgrass (Denchev et al. 1997). Bialaphos was successfully applied in barley (Koprek et al. 1996), maize (Brettschneider et al. 1997), sorghum (Casas et al. 1997), sugarcane (Gallo-Meagher and Irvine 1996), and wheat (Takumi and Shimada 1997). Many more transformation events reported in recent years reveals that the herbicide selection method is very useful for monocots.

Both antibiotics and herbicides are destructive selection agents, because in young developmental stages, even the resistant cells can be killed if the applied concentration is too high. Therefore, the choice of a nondestructive selection method can be a good help. The firefly (*Photinus pyralis*) luciferase gene is the most useful nondestructive selectable gene, which catalyzes the emission of luminescent light with the presence of luciferin (Ow et al.1986). The luciferase activity can be checked periodically through all developmental stages without damaging the assayed samples.

In conclusion, all the modern genetic modification tools can be applied to *Alstroemeria*, but up to now there is no report about a successful *Alstroemeria* transformation protocol.

Outline of the thesis

In this thesis several investigations have been made to develop a good regeneration system applicable for micropropagation and for genetic transformation in *Alstroemeria*. There were two plant regeneration systems involved in this study. One based on organogenesis and the other one on somatic embryogenesis. The transformation studies were mainly focused on particle bombardment. In Chapter 2 to 4 plant regeneration based on organogenesis via leaf explant culture is described, and in Chapter 5 somatic embryogenesis procedures using callus culture initially induced on stem segments are described. Chapter 2 reveals the development of a two-step regeneration system by using excised leaf explants as initial plant material. The culture conditions, the efficiency of shoot induction from leaf explants, and the subsequent development of shoots into complete plants with rhizomes are investigated. Chapter 3 reports the origin of the regenerating shoots on the leaf explants by using histological methods. Also the regeneration capability of leaf explants from different stem positions is compared. In Chapter 4, the multiplication efficiency of leaf culture and the traditional rhizome culture is compared. The flowering plants derived from the different propagation methods are grown in the greenhouse for comparison. The application of leaf culture protocol to existing cultivars is discussed. Chapter 5 describes the development of a plant regeneration system based on callus culture. The whole regeneration system from callus induction, proliferation, maintenance, somatic embryogenesis, and plant formation is given in this chapter. In chapter 6, the recovery of transgenic plants via particle bombardment is described. Two plasmids containing destructive and nondestructive selection genes respectively, are used, and the selection efficiency is discussed. Chapter 7 is a general discussion, the advantages and disadvantages of the two plant regeneration systems, leaf explant culture and callus culture, are compared, and the possibilities and restrictions of the application of particle bombardment mediated transformation in future *Alstroemeria* breeding is discussed.

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Chapter 2

Direct shoot regeneration from excised leaf explants of *in vitro* grown seedlings of *Alstroemeria* L.

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Abstract. A two-step protocol for the induction of shoots from *Alstroemeria* leaf explants has been developed. Leaf explants with stem node tissue attached were incubated on shoot induction medium for 10 days, and then transferred to regeneration medium. Shoots from the area adjacent to the region between leaf base and node tissue regenerated within three weeks after transfer to the regeneration medium, without a callus phase. The best induction was obtained with Murashige and Skoog (MS) medium containing 10 μM thidiazuron (TDZ) and 0.5 μM indole butyric acid (IBA). The regeneration medium contained 2.2 μM 6-benzylaminopurine (BAP). After several subcultures of the leaf explants with induced shoots, normal plantlets with rhizome were formed. In *Alstroemeria* the percentage of responding leaf explants is more important than the number of shoots regenerated per leaf explant, because rhizome formation is the most important factor for micropropagation. The effect of other compounds in the induction medium, including glucose, sucrose, silver nitrate, and ancymidol, on regeneration was also investigated.

Key words: *Alstroemeria*, Regeneration, Thidiazuron(TDZ), Silver nitrate, Ancymidol

Abbreviations: *BAP* -6-Benzylaminopurine, *IBA* -Indole butyric acid, *MS* Murashige and Skoog, *TDZ* Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-yl urea)

Introduction

Alstroemeria, an endemic Latin American monocot cutivated for its flowers, has become increasingly popular in recent years. Important reasons are the diversity of colours, low energy requirement and long vase life, which allow competition with other greenhouse-grown cut flowers. Generally, *Alstroemeria* is propagated vegetatively by rhizome division, but the propagation rate is rather low. Therefore micropropagation based on rhizome cuttings or rhizome meristem culture has been developed to accelerate the multiplication efficiency (Gabryszewska and Hempel 1985, Hakkaart and Versluijs 1988, Pierik et al. 1988, Van Zaayen et al. 1992, Bond and Alderson 1993). Flower pedicels, subapical segments from the vegetative

stem, and rhizome tips of *Alstroemeria* were tested by Lin et al. (1987) as initial explants for tissue culture, of which rhizome tips gave the best response. Recently, plant regeneration via somatic embryogenesis has been reported following the induction of callus from mature (Gonzalez-Benito and Alderson 1992, Hutchinson et al. 1994) or immature (Van Schaik et al. 1996) zygotic embryos of *Alstroemeria*. Other plant tissues of *Alstroemeria*, however, have not yet been reported as initial explants for *in vitro* culture. In this report, a new protocol for *Alstroemeria* micropropagation via leaf explant culture is presented after investigation of the influences of thidiazuron (TDZ), sucrose, glucose, silver nitrate and ancymidol in the shoot induction medium.

Material and methods

Plant material

Selfed seeds of the *Alstroemeria* genotype VV024 (a tetraploid breeding line from van Staaveren BV, The Netherlands), were surface sterilized in 70% ethanol (1 min), 3% sodium hypochlorite solution (20 min) and then rinsed three times with sterilized water (10 min each). The seeds were incubated in test tubes with half strength Murashige and Skoog medium (1962) (MS), 1% sucrose and 0.7% micro agar (0.5MS10 medium, pH 5.8). After germination, the seedlings were subcultured four times in 0.5MS10 medium every four weeks for the production of erect shoots, and finally the plants were maintained on MS medium containing 2.2 μM 6-benzylamnopurine (BAP). Cultures were placed under 18°C, and 12 h light.

General Protocol for leaf explant culture

Fully developed leaves with attached stem nodes were cut from the *in vitro*-grown erect shoots and used as explants. After cutting off the upper part of the leaf blade, the explants were placed on culture medium with the abaxial side touching the medium. In each experiment the five top leaves were used. All experiments were carried out by two-step procedures. First, the leaf

explants were cultured on shoot induction medium for 10 days, and then they were transferred to regeneration medium. Based on pilot studies the period of 10 days for induction was optimal.

MS medium was used as basal medium in all experiments, and the pH was adjusted to 5.8 before autoclaving. The standard shoot induction medium contained 6.9 μM TDZ, 0.5 μM indol butyric acid (IBA), 3% glucose, and was solidified with 0.74% Daishin agar. The standard regeneration medium contained 2.2 μM BAP and was solidified with 0.7% micro agar.

Our experiments were concentrated on improving the leaf culture by testing different cutting methods and by varying the composition of the standard induction medium. Ten leaf explants were cultured in each petri dish and five replicates were prepared for each treatment. The cultures were incubated at 18°C in the dark in both induction and regeneration medium.

Cutting methods for leaf explants

To compare the effect of the size of the attached stem tissue, two cutting methods were tested. (Fig. 1; the arrows indicate the direction of cutting). The leaf explant prepared by the C1 method carried a larger amount of stem tissue than that prepared by the C2 method. When prepared according to the C2 method across the nodal portion, the lower part of the stem tissue was not involved.

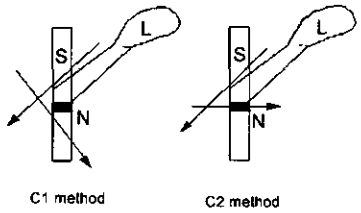

Fig. 1. Cutting methods for leaf explants of *Alstroemeria*.
Arrows indicate the direction and place of cutting.
S:stem, L:leaf, N:node.

Concentrations of thidiazuron (TDZ) in combination with glucose or sucrose in induction medium

Based on pilot studies, TDZ seemed to stimulate shoot induction: medium without TDZ had no effect, 16 μM had a toxic. Therefore, the basal medium supplemented with TDZ (2, 4, 8, 10

μM), IBA(0.5 μM) in combination with glucose (3%) or sucrose (3%) was tested as an induction medium. Leaf explants following the C2 cutting method were incubated on induction medium for 10 days and then transferred to the standard regeneration medium.

Silver nitrate and ancymidol in the induction medium

Silver nitrate (0, 30, 60, and 120 μM) and ancymidol (0, 0.1, 1, and 10 μM) were supplied to the induction medium (MS+10 μM TDZ+0.5 μM IBA+3% sucrose) to investigate their influence on regeneration. The C2 method and the standard regeneration medium were used in this experiment. The percentage of regenerating shoots and the number of shoots per regenerating explant were recorded 4 and 8 weeks after transfer to the regeneration medium. Analysis of variance was performed on data collected using the LSD test at the 5% level.

Results

Shoot formation on the leaf explant and the influence of the cutting method

After 10 days incubation on the shoot induction medium, the leaf explants remained green and two types of response were found: (1) the leaves turned dark green without a further growth response and did not form shoots after subculture in regeneration medium; (2) the leaf explants showed elongation of the petiole and enlargement of the nodal section, which were able to form shoots after subculture onto regeneration medium.

Direct shoot formation was observed in the area adjacent to the region between leaf and stem, and no callus was formed. Buds accompanied by leaf primodia (Fig. 2a) were found inbetween leaf blade and stem node after 3 weeks of subculture in regeneration medium. Two weeks later the first leaf expanded and the main shoot elongated (Fig. 2b).

The complete leaf explants with all the newly formed shoots were subcultured in the standard regeneration medium for about 2 months, and the shoots developed into normal plantlets with rhizome tips and roots (Fig. 2c). Subculturing the shoots alone after excising them

from the leaf explants was not successful; only shoots with rhizome tips were able to survive after separation and subculture.

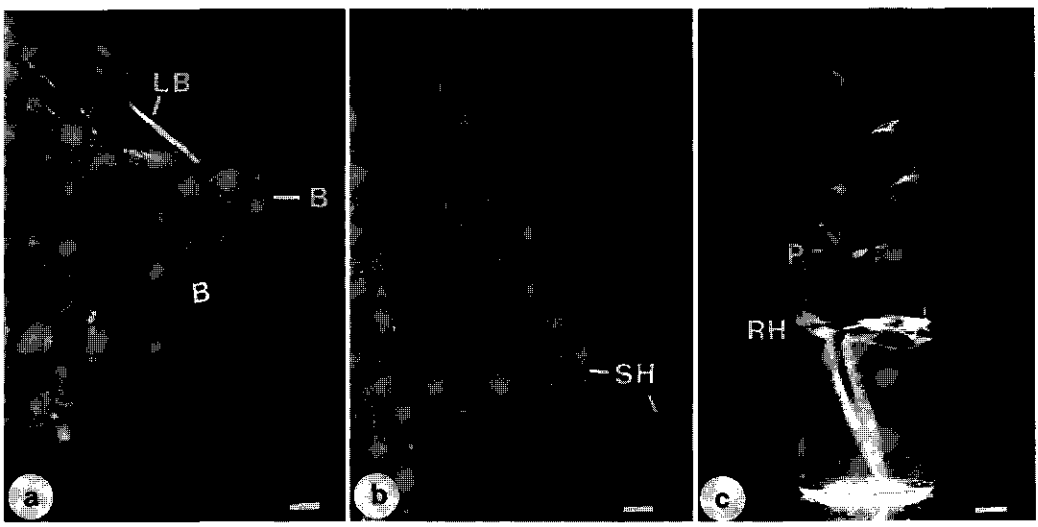

Fig. 2. Shoot induction and regeneration at the leaf explant of *Alstroemeria*. a. Buds regenerated from adjacent area between leaf base and stem node after 3 weeks of subculture on regeneration medium. (Bar=1 mm) b. Shoot cluster developed on a leaf explant after 5 weeks of subculture on regeneration medium. (Bar=2.5 mm) c. Plantlets regenerated after two months of subculture. (Bar=5 mm) B:bud, LB:leaf base, P:plantlet R:root, RH:rhizome, S:stem, SH:shoot.

Table 1 presents the effect of two cutting methods. After 4 weeks of subculture on regeneration medium, the leaf explants cut by the C2 method showed 18% of shoot regeneration, higher than that of the C1 method (8%). There was no difference between C1 and C2 methods in the number of shoots per explant.

Table 1. Effect of different cutting methods on shoot regeneration of *Alstroemeria* leaf explants. The cutting methos is illustrated in Fig.1.

Treatment	Total number of leaf explants	Explants with shoot formation	Number of shoots per regenerating explant
C1	50	4 (8%)	2.0±0.7
C2	50	9 (18%)	1.6±0.7

The influence of TDZ concentration and different kinds of sugar

The concentration of TDZ in the shoot induction medium significantly influenced the response of the leaf explants. The percentage of shoot formation progressively increased with increasing concentrations of TDZ from 2 μM to 10 μM; 10 μM showing the highest response (Table 2). TDZ at 10μM induced threefold more regeneration than 2 μM. Comparing the results of 4 weeks with those of 8 weeks, higher concentrations of TDZ not only induced a higher percentage of shoot, but also resulted in earlier development of shoot formation. However, there was no significant difference between sucrose or glucose treatment (Table 2). For all treatments the mean number of shoots per regenerating explant ranged from 1.9 to 3.9, and no prominent differences were found between the treatments.

The effect of silver nitrate and ancymidol on shoot regeneration

The addition of silver nitrate, 30-120 μM, in the induction medium did not increase the shoot regeneration rate. There was no significant difference between 30 μM silver nitrate and control treatment, while higher concentrations (60 μM and 120μM) significantly reduced the percentage of shoot regeneration (Table 3). The average number of shoots induced on regenerating explants ranged from 2.5 to 3.7 (Table 3). There were no differences among the treatments.

The effect of ancymidol in the induction medium on shoot regeneration is also presented in Table 3. Ancymidol had no positive effect on the percentage of shoot regeneration. However, the average number of shoots per explant, was increased slightly with an increasing ancymidol concentration.

Table 2. Effect of TDZ concentration and sugar composition in the induction medium on shoot regeneration of *Alstroemeria*. Leaf explants were incubated on shoot induction medium for 10 days, then subcultured on regeneration medium. Each value was recorded 4 and 8 weeks after subculture. Values followed by different letters are significantly different at the 5% level as determined by LSD (*NS* not significalt).

TDZ	4 week-cultures [a]		8 week-cultures	
(μM)	Shoot regeneration (%)	Number of shoots per regenerating explant	Shoot regeneration (%)	Number of shoots per regenerating explant
Sugar=3% sucrose				
2	4 b	1.3±0.5	18 c	2.8±1.8
4	4 b	1.0±0	28 bc	2.0±1.1
8	28 a	2.0±0.6	40 b	1.9±0.6
10	36 a	1.8±0.8	56 a	1.8±0.7
Sugar=3% glucose				
2	8 b	3.5±2.5	12 b	3.5±2.5
4	24 a	3.6±2.0	32 a	3.9±1.9
8	20 a	4.3±0.6	36 a	3.0±2.0
10	36 a	1.9±0.7	40 a	1.9±0.8

Factor (% of shoot regeneration)		Significant level	
		4 week-culture	8 week-culture
Sugar		NS	NS
TDZ concentration	p<0.003	p<0.002	
Sugar x TDZ		NS	NS

Table 3. The effect of silver nitrate ($AgNO_3$) and ancymidol in induction medium on shoot regeneration from leaf explants of *Alstroemeria*. Leaf explants were incubated on shoot induction medium for 10 days, then subcultured on regeneration medium. Each value was recorded 4 and 8 weeks after subculture.

Treatment[a]	4 week-cultures [b]		8 week-cultures	
(μM)	Shoot regeneration (%)	No. of shoots per regenerating explant	Shoot regeneration (%)	No. of shoots per regenerating explant
$AgNO_3$				
0	38.0 a	2.4±0.5	56.0 a	3.7±0.8
30	27.5 a	3.0±0.9	62.5 a	3.4±0.6
60	12.0 b	2.0±0.8	26.0 b	2.5±0.7
120	12.0 b	2.2±1.2	30.0 b	2.9±1.0
ancymidol				
0	22.0 a	2.4±0.4	22.0 a	3.6±0.6
0.1	20.0 a	2.0±0.4	24.0 a	2.4±0.6
1.0	30.0 a	3.3±0.5	34.0 a	4.4±0.8
10.0	24.0 a	3.8±1.7	28.0 a	4.8±1.8

[a] $AgNO_3$ and ancymidol were supplemented to the induction medium (MS+10.0 μM TDZ+0.5μM IBA+3% sucrose) respectively.

[b] Mean separation in columns followed by different letters are significantly different at 5% level as determined by LSD.

Discussion

Alstroemeria plantlets were produced via the organogenesis procedure and for the first time leaf explants were successfully used as initial material. We were able to induce shoots directly from excised leaf explants with high frequency, and the shoots subsequently developed into normal plantlets. It was shown that leaf blades with adhering stem node tissue, which were easily cut from erect shoots without damaging the rhizome tips, were good initial explants for micropropagation. This type of shoot regeneration is similar to that found in dicotyledonous flower crops such as *Dianthus* (Van Altvorst et al. 1994), but is unique for monocots. In *Anthurium* (Kuehnle et al. 1992), *Iris* (Jehan et al. 1994), *Secale* (Jia & Zhang 1993), and *Zingiber* (Kackar et al. 1993), leaves without attached stem tissues were used as initial material, and plantlets were regenerated indirectly via embryogenic callus. In our experiments, no callus formation was found. So we developed a direct regeneration system through leaf culture.

Thidiazuron (TDZ), a cytokinin-like substance, has been effectively used in inducing shoot regeneration on leaf explants of many dicots (Huetteman & Preece 1993; Dubois & de Vries 1995; Turk et al. 1994), but it has not been reported in monocots. In *Alstroemeria*, TDZ (0.5 μM) in combination with BAP (8 μM) was able to induce multiple shoot formation from embryo-induced callus (Hutchinson et al. 1994). In the present report TDZ was an important factor in shoot induction medium for *Alstroemeria* leaf culture. The concentration of TDZ greatly influenced the frequency and the speed with which shoots were formed, and 10 μM of TDZ proved to be a suitable concentration.

For comparing multiplication efficiency among different treatments, the percentage of response is more important than the number of shoots regenerated per leaf explant. Because rhizome formation is very important in *Alstroemeria*, the separation of individual shoots without rhizome resulted in the death of shoots. Therefore, in an early developmental stage of regeneration (in 8-week-old cultures) the whole leaf explant together with the newly formed shoots has to be treated as one propagation unit, and the number of shoots per leaf explant cannot be regarded as a multiplication index.

The size of stem tissue attached to the leaf explant may affect the regeneration frequency and the number of shoots induced per explant. In carnation leaf culture, leaves with a larger amount of adhering stem tissue showed a much higher frequency and formed more shoots (Van Altvorst et al. 1994). In our experiments, however, leaf explants prepared by the C2 cutting method adhered to a smaller portion of stem tissue and gave rise to a higher frequency of regeneration than the C1 method. Leaf explants cut by C1 or C2 method showed no differences in number of shoots per regenerating explant.

Excised *Alstroemeria* leaves were rapidly turn yellow and senesce, which could be the effect of ethylene. Many ethylene antagonists have been developed to prevent leaf yellowing of cut *Alstroemeria* flowers (Serek et al. 1995; Van Doorn & Van Lieburg 1993). Endogenous and exogenous ethylene may play an important role in the regeneration of cultured tissues, either as a promoter or as an inhibitor (Biddington 1992). In leaf cultures of *Prunus* (Escalettes & Dosba 1993) and *Rosa* (Dubois & de Vries 1995), adding silver nitrate (an ethylene antagonist) in regeneration medium enhanced shoot regeneration. In our experiments, 30-120 μM of silver nitrate in the induction medium did not enhance shoot regeneration from leaf explants of *Alstroemeria*. Perhaps the concentration range used in this experiment was too wide, because no

difference was found between control and 30 μM (lowest concentration) treatment, and 60 μM and 120 μM (higher concentrations) suppressed shoot regeneration.

The growth retardant ancymidol has been used to promote shoot formation from nodal segments (Chin 1982) or shoot apices (Kohmura et al. 1994) in *Asparagus*. This promoting effect was also found in *Alstroemeria* leaf culture, inducing a higher number of shoots per explant, but it did not promote the percentage of responding explants.

In this report we presented a new protocol for regenerating shoots from leaf explants of *Alstroemeria*. As the commonly used micropropagation method is based on rhizome multiplication, the erect shoots with leaves were cut off and discarded. It is expected that the propagation efficiency with *Alstroemeria* could be accelerated by using leaf explant as another micropropagation initial in combination with rhizome multiplication.

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Chapter 3

Formation of shoots from leaf axils of *Alstroemeria*: The effect of the position on the stem.

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Abstract

Direct shoot regeneration was induced from leaf explants of *Alstroemeria*. The explants contained a leaf blade and a small portion of stem node, which were taken from the erect shoots of *in vitro* multiplicated plantlets. The shoot regeneration capacity of the excised leaf explants was significantly related to the position of the explant on the stem. The youngest explant which located close to the shoot apex gave the highest response. A gradient response towards the shoot apex was observed in percentage of shoot regeneration and in the number of shoots per regenerating explant. Histological studies revealed that shoots were initiated at the leaf axils. The origin of the adventitious bud was located at the epidermal layer of stem peripheral cells.

Key words: age, *in vitro*, Inca lily, monocots, micropropagation, regeneration

*Abbreviations: B*AP -6-benzylaminopurine, IBA -indol-3-butyric acid, MS medium -Murashige Skoog's (1962) medium, TDZ(thidiazuron) -N-phenyl-N'-1,2,3-thidiazol-5-yl urea

Introduction

Alstroemeria is a monocotyledonous ornamental plant. It is vegetatively propagated but with a low multiplication rate. To accelerate propagation, *in vitro* culture techniques based on rhizomes have been developed (Pierik et al. 1988; Bond & Alderson 1993), but the multiplication rate was still low. Furthermore, flower pedicels and stem segments have been tested but they showed a low response (Lin et al. 1987). Zygotic embryos were successfully used to induce embryogenic callus in high frequencies (Gonzalez-Benito & Alderson 1992; Hutchinson et al. 1994; Van Schaik et al. 1996), but this approach cannot be used for propagation of existing cultivars. We have previously reported a protocol for inducing plantlets from cultured leaf explants of *in vitro* grown seedlings (Lin et al. 1997). In this study, we examine the effect of the position of leaf explant on the stem. The origin of the shoots and their developmental process was examined in a histological study.

Materials and methods

Plant material and preparations of leaf explant

An *Alstroemeria* clone, VV2406, was selected from selfed progenies of VV024 (a tetraploid breeding line from Van Staaveren BV, Aalsmeer, The Netherlands) and multiplicated *in vitro*. Shoots of approximately 10 cm in length with five fully developed leaves were collected and shoot apices were removed. The period of shoot development from the bud stage to the five-leaf stage took about 6 weeks (data not shown). The first five fully developed leaves with adhering node tissue were cut from the stem, numbered from top to bottom as 1 till 5, and transferred to the medium.

The two-step regeneration procedure described by Lin et al. (1997) was used: leaf explants were incubated for 10 days on inducing medium (MS 10 μM TDZ 0.5 μM IBA 3% sucrose), and then transferred to shooting medium (MS 2.2 μM BAP 3% sucrose). All cultures were incubated in 9-cm Petri dishes at 18°C in the dark. Ten leaf explants were cultured in each Petri dish and four replicate dishes were prepared for each treatment.

To analyze the shoot regeneration capacity, the percentage of regeneration and the mean number of shoots per regenerating explant were recorded 4 and 8 weeks after subculture respectively. Mean separation was performed by the least significant difference (LSD) test (Snedecor & Cochran 1994) at 5% level.

Histological research

To investigate the origin of the newly formed shoots, and the timing of developmental events, explants were collected after 0 (CK) and 10 days on inducing medium, and after 7, 14, and 21 days on shooting medium. Ten explants were collected in each stage.

Explants were fixed in 5% glutaraldehyde solution (in 0.1 M potassium phosphate buffer, pH 7.2) for 2 hours at room temperature, rinsed in buffer, dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer GmbH). Serial sections of 5 mm were made by

using the Reichert-Jung Autocut 2055 microtome. Sections were stained with 0.25% toluidine blue on glass slides and were photographed under bright field with Zeiss Axiophot microscope.

Results

The influence of position on the stem

The detached leaves remained green and had elongated after 10 days of culture on inducing medium in dark conditions. No visible differences between leaf explants, which were taken from different positions of the stem, were observed. After three weeks of subculture on shooting medium, buds had developped from the region between stem and leaf base were observed. Leaf explants without regenerating shoots turned gradually brown.

Four weeks after subculture on shooting medium the leaf explants with or without regenerating shoots could easily be distinguished, and significant differences between nodal positions were observed. The younger explants, which originally located closer to the stem apex, showed a higher percentage of shoot regeneration than the older explants, and this percentage significantly decreased with increasing positions. A similar gradient response was also found in the number of shoots per regenerating explant. Younger explants produced more shoots than older explants (*Table 1*).

After incubation for another four weeks, the responding explants continuously formed new shoots. Some of the explants did not produce shoots during the first four weeks but they were able to produce shoots after this period. However, the explants without regenerating ability turned brown and died. The explants showed their greatest regenerating potential after eight weeks of subculture, and explants taken from different positions showed a similar response (*Table 1*). After eight weeks of subculture, the first (youngest) explants produced estimatedly 120 times more shoots than the fifth (oldest) (*Table 1*).

Table 1. The effect of the position on the stem on shoot regeneration of leaf explants of *Alstroemeria* clone VV2406. Leaf explants were incubated on inducing medium for 10 days, and then subcultured on shooting medium. Each value was recorded 4 and 8 weeks after subculture on shooting medium. [1]

	4-week culture		8-week culture		
Nodal position [2]	Shoot regeneration percentage	Number of shoots per regenerating explant	Shoot regeneration percentage	Number of shoots per regenerating explant	Shoot regeneration capacity per 100 leaves[4]
1	82.5 a [3]	4.5 a	95.5 a	6.9 a	659
2	70.0 ab	4.0 a	85.5 ab	4.7 a	402
3	52.5 b	2.4 b	67.5 b	3.0 b	203
4	20.0 c	1.8 b	32.5 c	2.7 b	88
5	0.0 d	0.0 c	5.0 d	1.1 c	6

[1] Inducing medium is MS(1962)+10 μM TDZ+0.5 μM IBA+ 3% sucrose, and shooting medium is MS+2.2 μM BAP.

[2] The nodal position of the leaf explant was numbered as 1,2,3,4,5 from top to bottom, while the shoot apex was removed.

[3] Mean separation within columns by LSD test, values followed by different letters are significantly different from each other at the 5% level.

[4] Shoot regeneration capacity per 100 leaves = shoot regeneration percentage x number of shoots per regenerating explant x 100.

Histological analysis

Histological sections of the intact explants showed that cells of leaf and stem tissue were similar in shape and size. Vascular bundles were present in the leaf tissue and scattered all over the stem tissue. Cells at the epidermic layer of leaf and stem tissue appeared to be smaller in size and more compact than the surrounding parenchyma cells. No initials of axillary buds or meristematic structures have ever been found at the epidermic layer of leaf, stem and transitional tissues (*Figure 1*).

After 10 days of incubation on inducing medium the leaf and stem tissues became swollen and the cells enlarged. At the transition layer, however, individual epidermic cells were initiated to divide. The dividing cells were located at the epidermal layer of the stem part. They were smaller in size than the surrounding cells and contained densely stained nuclei (*Figure 2*). After several divisions the cells formed a meristem-like structure (meristemoid), which appeared in the leaf axils (*Figure 3*).

After subculture of the explants on shooting medium for seven days the meristemoid developed into a meristematic structure. The meristematic structure had a broad base and a protrudent apex with vigourously dividing cells, and numerous newly developing meristemoids were observed at the basal region as well as at the leaf epidermis (*Figure 4*). After 14 days on shooting medium the meristematic structure developed into a small bud and this bud protruded out of the original initiated tissues (*Figure 5*). After 21 days on shooting medium the bud developed into a shoot apex, with an apical meristem and a leaf primordium (*Figure 6*).

Discussion

Nodal leaf explants taken from in vitro grown seedlings were found to be good starting material for *Alstroemeria* micropropagation (Lin et al. 1997). This report demonstrated that leaf explants taken from *in vitro* multiplicated plantlets were also able to regenerate shoots with a high percentage. Therefore, juvenile material (from seedlings) is not a necessary requirement, so that this protocol could be used for propagating existing cultivars.

The age of cultured explants, in some plant species such as *Geranium* and *Brassica*, is a crucial factor in affecting the regeneration ability (Chang et al. 1996; Choi et al. 1996). Silvertand et al. (1995) and Slabbert et al. (1995) have reported another type of age-related effect in monocots of *Allium* and *Crinum* respectively. The length of young flower stalks, which is related to the physiological age, influenced the percentage of adventitious bud formation. Our results showed that the position of explants on the stem has a great influence on the regeneration ability. This could be a kind of age-related response, because the position was related to the physiological age of the explants, and the closer to the apex the younger the explant.

A gradient regenerating response towards the shoot apex was observed, and the closer (younger) to the apex the higher the response. This gradient response was found in carnation (dicots) leaf culture (Van Altvorst et al. 1995), but has not been reported in monocots. For callus initiation, however, a gradient response was observed in leaf culture of some monocots such as *Hordeum* (Becher et al. 1992), *Miscanthus* (Holme & Petersen 1996), and *Avena* (Chen et al.

1995). The physiological age effect was also found in flower stalk cultures of *Allium* (Silvertand et al. 1995) and *Crinum* (Slabbert et al. 1995). The relation of the younger the stalk the higher the response was clearly found.

Stem nodes (with or without apical meristem) were found to be very useful explants for initiating micropropagation systems in some monocots. The shoots were induced directly, without a callus phase, from pre-existing axillary buds (Swamy et al. 1983; Tisserat 1984), or from newly formed adventitious buds (Hwang et al. 1984; Duan & Yazawa 1995; Nakamura & Hattori 1995), or from both (Meyer & Van Staden 1991; Pandey et al. 1992). Our experiments in *Alstroemeria* showed a direct bud formation without a callus phase. It is a kind of adventitious bud regeneration, because there is no pre-existing axillary bud or bud-primordium been found. The adventitious buds were induced from individual epidermic cell of the leaf axil tissues, and it seems that the cells within leaf axils exibit a high potential for regeneration. The pattern of shoot development, from a single cell to a complete shoot, was similar to that of rice (Nakamura & Hattori 1995). However, the starting material was different. In rice they used germinating seeds in stead of excised leaf explants. On the other hand, although in both cases the adventitious buds were induced from the epidermal cells, the subepidermal cells were also involved in rice were not included in *Alstroemeria*.

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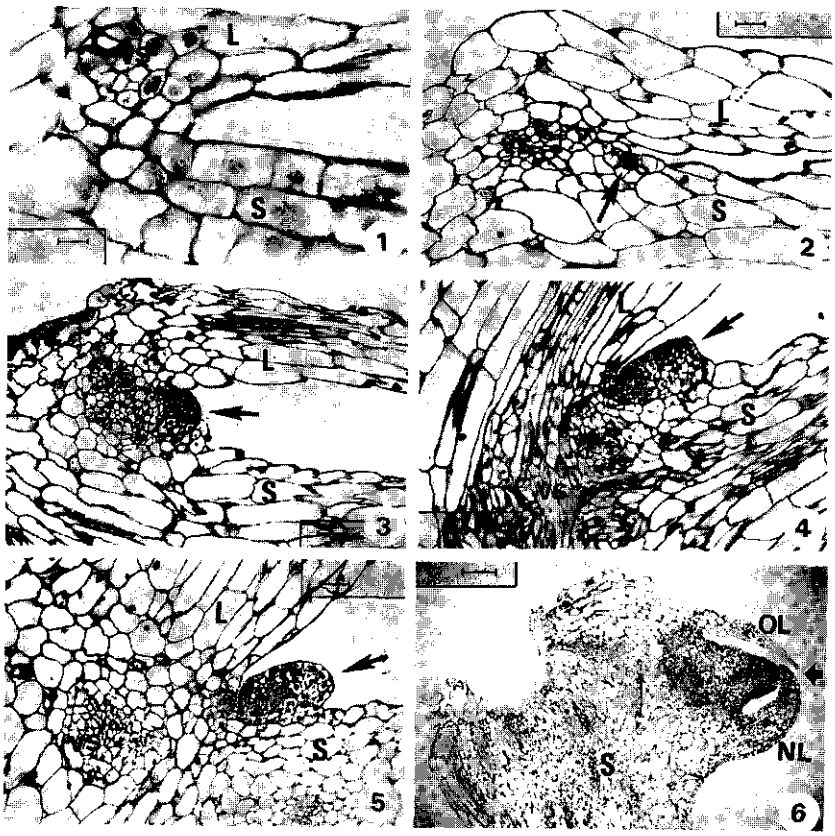

Figure 1-6. Developmental sequence of the regeneration of adventitious shoots on leaf axils of *Alstroemeria* L. The leaf explant was incubated on inducing medium (MS+10 μM TDZ+0.5 μM IBA) for 10 days, then subcultured on shooting medium (MS+2.2 μM BAP) for 21 days. (S=stem tissue; L=leaf tissue; VS=vascular bundle). *Figure 1.* The original status of stem and leaf tissues at day 0. *Figure 2 & 3.* After 10 days of incubation on inducing medium, in *Figure 2* the arrow indicates epidermal cell divisions, and in *Figure 3* the arrow indicates a meristemoid structure with densely staining cells. *Figure 4.* After 7 days of subculture on shooting medium. Arrow indicates meristematic structure, and arrow head indicates more initiating meristemoids. *Figure 5.* After 14 days of subculture on shooting medium. Arrow indicates newly formed adventitious bud. *Figure 6.* After 21 days of subculture on shooting medium. Arrow indicates shoot apex with meristem in the center together with the newly formed primordial leaf (OL: original leaf, NL: the new leaf primordium.) Scale bar=25 mM for *Figure 1*, 100 mM for *Figure 2,3,4,5*, and 400 mM for *Figure 6*.

Chapter 4

The application of leaf explant culture protocol in enhancing the multiplication efficiency of *Alstroemeria*.

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Abstract

Six tetraploid *Alstroemeria* selfed progenies were propagated *in vitro* by rhizome multiplication, whereby the shoots were cut off from the rhizome and discarded. In a three-weeks subculture interval, the average rhizome multiplication rate was 2.3. The discarded shoots could be used to initiate another propagation system. When using leaf explants excised from the shoots, a cyclic mass propagation system was established. The first three leaves, excised from each shoot, have an average regeneration capacity of 87.7%, and the number of shoots per explant was 5.3. Combining the rhizome multiplication with the leaf explant culture, the multiplication efficiency was enhanced. The plants were grown in the greenhouse to maturity. Plants regenerated from leaf explants were morphologically identical to those originated from rhizome multiplication. The leaf explant culture protocol was not only suitable for *in vitro* grown explants, but also applicable to *in vivo* grown explants.

Key words: Alstroemeria, Inca lily, micropropagation, monocots, rhizome

Introduction

Alstroemeria is a perennial crop mainly cultured for cut flower production. It is generally propagated by rhizome division. Due to the low multiplication rate and seasonal restrictions, several *in vitro* propagation systems were developed, based on rhizome tip and/or rhizome meristem multiplication (Gabryszewska 1995; Gabryszewska and Hempel 1985; Lin and Monette 1987; Hakkaart and Versluijs 1988; Pierik et al. 1988; Van Zaayen et al. 1992; Bond and Alderson 1993). In a four- week subculture interval the rhizome multiplication rate was between 2.0 to 3.0, depending on culture conditions and the genotype (Pierik et al. 1988). Compared to other plant species this rate is rather low. Since the rhizome tip was the best multiplication unit in *Alstroemeria* (Lin & Monette 1987) and other organs were difficult to regenerate into plants, the shoots usually were cut off and discarded during subculture. Lin et al. (1997) have developed a new micropropagation protocol by using leaf explants as propagation units. The adventitious buds, initiated from stem epidermis at the leaf axils,

developed into complete plants (Lin et al. 1997, 1998). This protocol provides an additional choice for mass propagation, and the multiplication efficiency is expected to be enhanced. Another aspect is the start of the rhizome culture from an *in vivo* grown plant. For this purpose usually the underground grown rhizome tip is used, whereby the disinfection is a major problem, which is difficult to overcome (Pedersen and Brandt 1992; Pierik et al. 1988). This problem may be solved by using the aerial grown leaves as starting material, because disinfection of an aerial plant tissue is expected to be easier than that of an underground part. In the present report a cyclic regeneration system based on leaf explant culture is presented. Morphological traits of flowering plants propagated by rhizome multiplication and by leaf explant culture are compared. Multiplication with leaf explants taken from *in vitro* and *in vivo* grown plant of an existing cultivar is also presented.

Materials and methods

Multiplication based on rhizome division

Selfed seeds of VV024 (a tetraploid breeding line from van Staaveren BV, Aalsmeer, The Netherlands) were sterilized and incubated on semi-solid half strength MS medium with 1% sucrose for germination (Lin et al. 1997). After germination the seedlings were kept on full strength Murashige and Skoog (1962)(MS) medium with 3% sucrose for four months. Afterwards the plantlets were transferred to MS medium supplemented with 0.5 mg/l BAP (BA0.5), and were constantly subcultured in a three-week interval for rhizome formation.

Two months after subculture on BA0.5 medium, many rhizomes were formed on each plantlet that were used for the multiplication experiments. In order to trace the multiplication behavior of this selfed population, each individual plantlets were subdivided into many parts. Each part contained a main rhizome, some developed aerial shoots, and other undeveloped axillary buds. After cutting off the aerial shoots, the axillary buds were kept, and the explants were transferred into test tubes with BA0.5 medium individually.

Six progenies were selected and 3 plantlets (replicates) for each progeny were used in this experiment. The number of developed and undeveloped buds, originating of rhizome or shoot,

were counted both before and after three weeks of culture. To evaluate the multiplication rate, the increased number of newly formed shoots and rhizomes was calculated. All the *in vitro* cultures were placed at 18°C and 12 h light.

Multiplication based on leaf explant culture and cyclic regeneration system

One *Alstroemeria* clone VV2406 was selected from the selfed progenies of VV024 for its high multiplication capacity. This clone was multiplicated and maintained *in vitro*. Shoots of approximately 5 cm in length with three fully developed leaves were collected and shoot apices were removed. The leaf explant culture protocol described by Lin et al. (1997) was used.

Ten leaf explants were cultured in each Petri dish and at least 10 replicates were prepared for each experiment. Eight weeks after incubation on regeneration medium the leaf explants together with the developing shoots were transferred to test tubes containing BA0.5 medium and were placed under 12 h light conditions. The subculture interval was four weeks. To develop a cyclic regeneration system, the newly formed shoots of about 5 cm were collected and the youngest three leaf explants were excised, and cultured on shoot induction medium for the next culture cycle. This way, three culture cycles were performed.

After the rhizomes, shoots, and roots were formed, 20 plants were transferred to the greenhouse. The morphological traits were measured and analyzed during the flowering period. The performance of plants derived from rhizome multiplication and leaf explant culture was compared.

Leaf explants cut from in vitro and in vivo grown plants

Alstroemeria 'CV118' (a tetraploid cultivar from Van Staaveren BV, Aalsmeer, The Netherlands) plants were subcultured *in vitro* on BA0.5 medium, and were used for collecting the shoots. The erect shoots of growth chamber grown (*in vivo*) 'CV118' plants were also collected. In order to compare the shoot regeneration ability, the leaf explants were excised from *in vitro* and *in vivo* grown shoots and were cultured on media according to the same procedures as mentioned above.

Before excision of the leaf explants, the *in vivo* grown shoots were sterilized by 3 % of sodium hypochlorite for 15 minutes and rinsed three times in sterilized water. The three fully developed top leaves were used in all preparations. Depending on the leaf size, either 10 leaves (*in vitro*) or 5 leaves (*in vivo*) were incubated in a petri dish and 5 replicates were prepared. Once the rhizome, shoots and roots were formed, the plants were transferred to the greenhouse.

Results

Rhizome multiplication

The rhizomic explant is a stem-like, horizontal growing, structure with 2-3 nodes and a sharp apex. Each node contains an upright growing shoot bud with an enlarged base. The rhizome apex, which appeared at one side of the former enlarged shoot base, is wrapped by a scale leaf and looks like a shoot bud. One week after subculture on BA0.5 medium, the rhizome apex elongats and becomes an upright growing bud. Two weeks later, this bud develops into a shoot with an enlarged base. Simultaneously, a new rhizome apex appears at one side of the shoot base. On the opposite site of the rhizome apex a root developed into the medium (Figure 1). The newly formed rhizome apex looked like an axillary bud of the first scale leaf of the upright growing shoot.

At the other nodes, one bud developed into a shoot and in some cases an axillary bud was formed at the other side of the shoot base. The axillary bud elongated and became a lateral rhizome tip subsequently. After three weeks the test tubes were filled with shoots and rhizome tips.

The results of shoot and rhizome multiplication in six selfed progenies of clone VV024 are shown in Table 1. In average, each explant produced 4.2 new shoots and 2.1 lateral rhizomes. A large difference of the mean number of shoots per explant was found among the progenies, ranging from 2.7 to 6.4. However, the mean number of lateral rhizomes per explant among the different progenies was quite the same, ranging from 1.3 to 2.5. In average, an original rhizome explant produced two times more shoots than lateral rhizomes in three weeks (Table 1.).

The average multiplication rate of shoots and rhizomes in the selfed population was 3.0 and 2.3 respectively, but a variation was found between the progenies (Table 1). The multiplication rate of shoots did not associate with that of the rhizomes, e.g., the progeny VV2454 had the highest shoot multiplication rate but had the lowest rhizome multiplication rate.

Table 1. The *in vitro* multiplication rates of six selfed progenies of VV024 using rhizomes as explants. Data were collected after 3 weeks' subculture of the rhizome explants on MS (1962) medium supplemented with 0.5 mg/l BAP.

Progeny	No. of shoots per explant	No. of lateral rhizomes per explant	Multiplication rate	
			shoot	rhizome
VV2406	4.2±0.8	2.2±0.3	2.5±0.5	1.9±0.3
VV2410	2.7±1.1	1.3±0.6	2.7±1.1	2.0±1.0
VV2434	4.3±0.5	2.5±1.3	3.6±0.8	3.3±1.7
VV2435	6.4±1.7	2.2±0.4	2.3±0.7	2.1±0.9
VV2452	4.0±2.0	2.2±2.2	3.2±2.2	2.8±1.5
VV2454	3.8±2.1	2.0±1.0	3.8±2.0	1.5±0.5
Average	4.2	2.1	3.0	2.3

Cyclic adventitious shoots regeneration system by using leaf exlants as a propagation unit

Adventitious buds were initiated from the region between the leaf base and stem node in four weeks of subculture of leaf explants on the regeneration medium. Eight weeks after subculture, the buds developed into shoots that were transferred to test tubes. The shoots kept growing in the test tubes and elongated. Two months later a rhizomic apex was initiated at the first node of a shoot. Once the rhizome was formed, individual shoots could be separated from the original leaf explant. After subculture for another month the rhizome developed into a complete plant with new shoots and roots.

From a leaf explant to a newly formed rhizome, a regeneration cycle was completed. One cycle took about 5 months. Once the newly formed rhizomes started to produce new axillary

shoots, the old shoots gradually turned yellow and died. Before yellowing, the old shoots were cut off and could be used for the next regeneration cycle. The second cycle was initiated by culturing the leaf explants excised from the first cycle's shoots.

In Table 2, the results of the regeneration system of three cycles are presented. The mean percentage of shoot regeneration ranged from 84.0 to 90.3% and there were no significant differences among the three cycles. The mean number of shoots per explant ranged from 4.9 to 5.9 and no significant differences were found. During the three cycles in culture the leaf explants always maintained a high regeneration ability.

Table 2. Three cycles of adventitious shoot regeneration in *Alstroemeria* clone VV2406 by using leaf explants as propagation units.

Cycle	Percentage of regeneration (%)	Number of shoots/explant
1	84.0±12.1	5.9±1.4
2	88.9±10.5	4.9±0.8
3	90.3±11.6	5.0±1.2
Average	87.7	5.3

Comparison of morphological traits of plants originated from rhizome multiplication and from leaf-explant culture

Plantlets of clone VV2406, which were multiplicated in vitro either by rhizome multiplication or by leaf explant culture, were successfully transferred to the greenhouse. In total, there were 19 and 15 plants survived, that were derived from rhizome multiplication system and leaf explant culture system, respectively. The plantlets originating from rhizome multiplication were larger in size than those from leaf-explant culture, having a higher survival rate (95% to 75%) and more shoots per plant (Table 3., Figure 2.). The rhizome multiplicated plants started to flower eight months after transfer to the greenhouse, however, the leaf-explant regenerated plants started to flower two weeks later. A number of morphological traits were measured and

listed in Table 3. All the vegetative and generative traits scored showed that plants multiplicated by the two different methods were morphologically identical (Figure 2).

Table 3. Comparison of morphological traits of *Alstroemeria* clone VV2406, which were propagated by two different multiplication systems: rhizome multiplication and leaf explant culture. The data were collected 8 months after transfer to the greenhouse, 19 plants from rhizome multiplication system and 15 plants from leaf explant culture system were measured.

Traits	Rhizome multiplication	Leaf explant culture
Vegetative parts		
No. of shoots/plant	14.2 ± 2.1	11.7 ± 3.0
Plant height (cm)	40.6 ± 5.2	43.5 ± 3.4
Leaf length (cm)	6.4 ± 0.6	6.2 ± 0.7
Leaf width (cm)	1.0 ± 0.1	1.1 ± 0.1
No. of leaves/shoot	17.0 ± 1.8	19.3 ± 3.7
Generative parts		
No. of flowers/shoot	12.8 ± 2.7	11.4 ± 2.3
No. of peduncles/shoot	5.1 ± 0.5	4.6 ± 0.5
Peduncle length (cm)	7.9 ± 1.5	7.0 ± 0.8
Flower length (cm)	5.1 ± 0.3	5.1 ± 0.3
Flower width (cm)	4.6 ± 0.4	4.4 ± 0.4
Flower color	pink	pink
No. of anthers/flower	6	6
Anther color	yellow	yellow
Pollen grain color	yellow	yellow

The application of leaf explant culture by using in vitro or in vivo grown shoots

The procedure of adventitious shoot formation on leaf explants of cultivar 'CV118' was similar to that of VV2406. Although the size of isolated leaf explants cut from *in vivo* grown plants was larger than that of *in vitro* plants, there were no significant differences between those two types of explants in the percentage of shoot regeneration and the number of shoots per explant (Table 4).

Leaf explants together with the regenerating shoots were subcultured in test tubes containing BA0.5 medium for two months, and the rhizomes were formed at the shoot base. It took 5 months from starting the experiment to get complete plantlets with shoots and rhizomes.

Once the rhizomes were formed, the plantlets were separated from the original leaf explants and subcultured on rooting medium. The whole plant including rhizome, shoots and roots was transferred to the greenhouse and they started flowering about six months after transfer. Visual comparison has been made and there were no morphological differences found between the plants derived from either *in vitro* or *in vivo* grown plants (Figure 3).

Table 4. Adventitious shoot regeneration from different explant sources of the tetraploid *Alstroemeria* cultivar 'CV118'.

Explant source*	Percentage of regeneration	Number of shoots/explant
In vitro	42.8±10.0	3.4±1.0
In vivo	37.8±3.9	5.2±3.2

* *In vitro* mean: leaf explants were taken from *in vitro* grown plants, which were originated from rhizome multiplication; *in vivo* means: leaf explants were taken from growth-chamber grown plants.

Discussion

Results of our rhizome micropropagation experiments are comparable to previous reports on multiplication of rhizome tips of *Alstroemeria* hybrids (Pierik et al. 1988). The variation of shoot and rhizome multiplication rate over progenies suggests a genotypic effect, due to the variated genetic background of the selfed progeny.

The rhizome is the main propagation organ in *Alstroemeria*. The aerial shoots grow out of the rhizome node and axillary rhizome buds are present at the base of each aerial shoot. Bond and Alderson (1993) suggested that a high apical dominance of the rhizome apex or aerial shoots is responsible for the suppression of the formation of lateral rhizomes. They found that the numbers of shoots were often not associated with the numbers of rhizomes. We also found this in our results, because in all tested progenies, the number of shoots per plant was not equal to the number of lateral rhizomes.

The time needed from transfer to the greenhouse to flowering, was longer for the leaf explant regenerated plants than for the rhizome multiplicated plants. This delay is probably not

due to the different propagation method, but to the different sizes of the plantlet. Pedersen et al. (1996) found that plant size might influence the time to flowering in *Alstroemeria*, and the larger the size, the earlier the flowering. In our experiments, the *ex vitro* plantlets produced by rhizome multiplication, were larger than those regenerated from leaf explants, which might be an explanation for earlier flowering.

The rhizome tip was previously found to be the only explant for initiating the *in vitro* multiplication system in *Alstroemeria* (Gabryszewska 1995; Lin and Monette 1987). Since the plants were grown in the soil, disinfection of underground rhizomes became a major problem and the infection due to internally present micro-organism was very difficult to overcome (Pierik et al. 1988). Thus a method for disinfection of rhizome tips was recommended by Pedersen and Brandt (1992), which contained two disinfectants and three disinfection steps. Lin et al. (1997) reported a micropropagation system by using *in vitro* grown leaf explants (including stem node) as initial material. In this paper we reported that this leaf culture technique was also useful by using *in vivo* (growth-chamber) grown leaf explants. Leaf explants, taken from aerial shoots, were easier to disinfect than underground rhizomes. Besides, some other advantages of this method were found: non-damage of the rhizomes, less disinfection steps, less infection problem, and the explants were easier to collect.

The conventional way of micropropagation for *Alstroemeria* is based on rhizomes. Therefore, the shoots are of no use and should be cut off during each subculture. This report has shown that both rhizomes and leaf explants can be used for plant propagation. A rhizome explant of *Alstroemeria* clone VV2406, for example, produced 2.2 rhizomes and 4.2 shoots for every 3 week (Table 1). Each shoot produced 3 leaf explants with an average regereration capacity of 87.7% (Table 2). Each leaf explant regenerated 5.3 shoots, which developed into plants within 5 months. Therefore, the multiplication efficiency was enhanced when combining both propagation systems.

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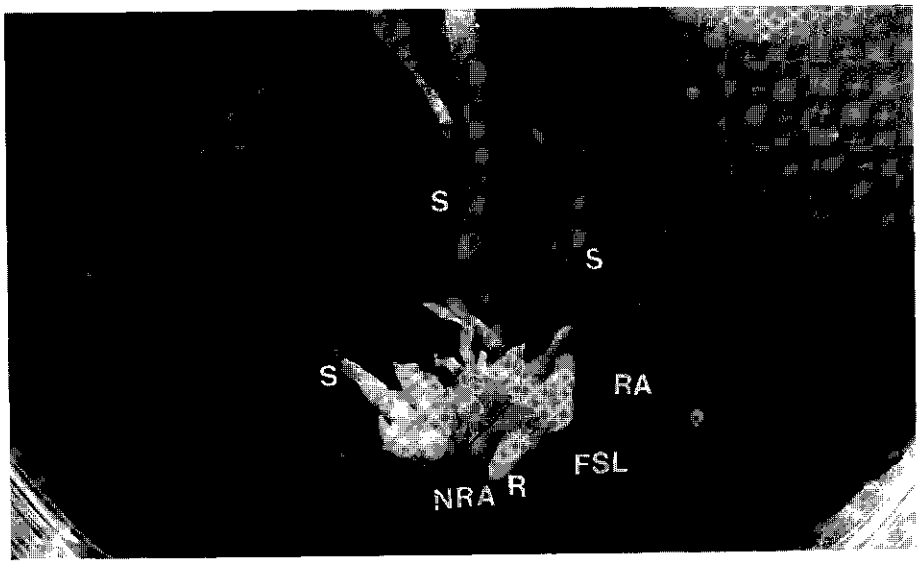

Figure 1. Rhizome plant of *Alstroemeria* VV2406 propagated *in vitro*. FSL first scale leaf, NRA newly-formed rhizome apex, R root, RA rhizome apex, S shoot, Bar = 0.5 cm

Figure 2. Flowering of Alstroemeria plant VV2406, 8 months after transfer to the greenhouse. A) originated from rhizome division, B) originated from leaf culture.

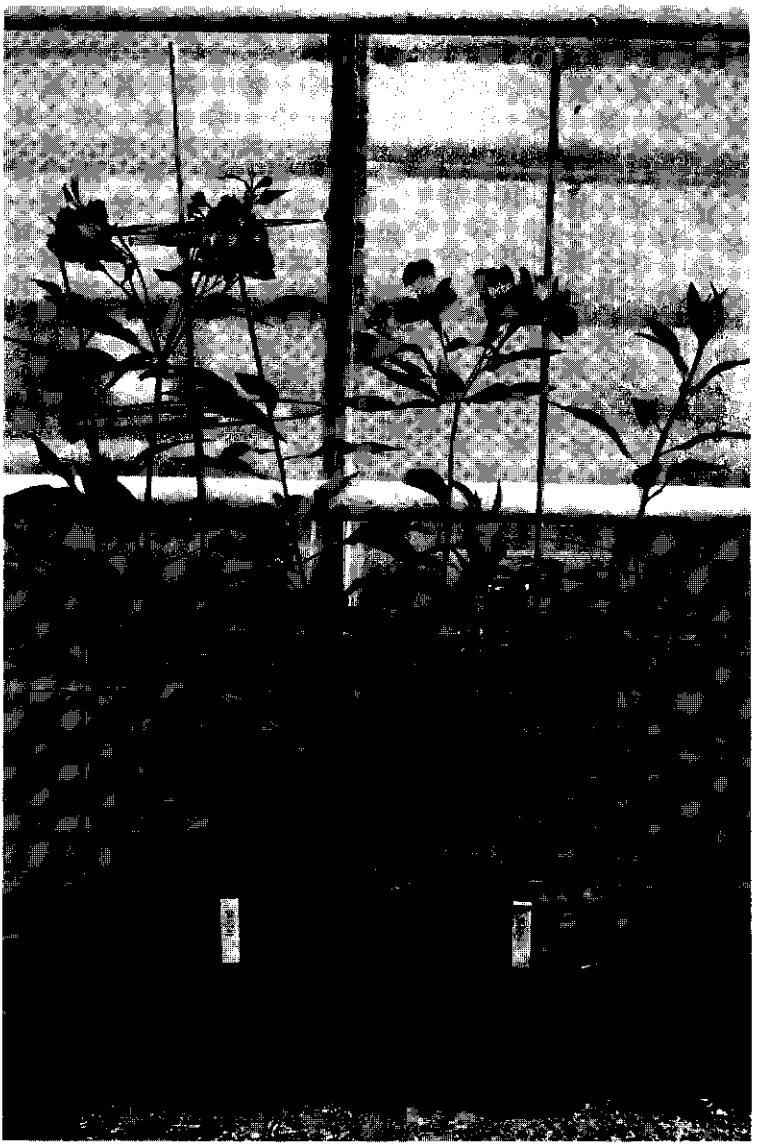

Figure 3. Leaf culture regenerated plants of *Alstroemeria* cultivar 'CV118' flowering in the greenhouse. The initial leaf explants were taken from either *in vitro* (left), or *in vivo* grown plants (right).

Chapter 5

Development of a plant regeneration system applicable for gene transformation in the ornamental *Alstroemeria*.

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Abstract

Stem segments of one-month old seedlings from two tetraploid *Alstroemeria* genotypes cultured on media supplemented with 4 mg/l 2,4-dichlorophenoxyacetic acid and 0.5 to 1.0 mg/l 6-benzylaminopurine initiated soft callus, which became compact after subculture on a medium with only 0.5 mg/l 6-benzylaminopurine. Subsequently, two different morphotypes of callus, friable and granular, were induced from the compact callus. The initiation, proliferation and maintenance of friable callus were accomplished on medium supplemented with 10 mg/l picloram. The granular callus was efficiently induced from compact callus if the medium contained 1 mg/l 2,4-dichlorophenoxyacetic acid and 0.5 mg/l 6-benzylaminopurine. In addition, the friable callus was able to differentiate into granular callus. The granular callus proved to be an intermediate between friable callus and somatic embryos. Friable and granular callus underwent somatic embryogenesis and plant regeneration on media supplemented with or without 6-benzylaminopurine. The total time needed from friable callus to a complete plantlet, with rhizome, shoots and roots, was approximately 6 months. This approach provides a cyclic system for the production of embryogenic material, which is considered to have valuable applications for genetic transformation in *Alstroemeria*.

Key words: callus, compact, culture, embryogenesis, friable, granular, monocots, regeneration, rhizome

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; MES, 2(N-morpholino) ethane sulfonic acid; MS, Murashige & Skoog (1962); NAA, α-naphthaleneacetic acid; pCPA, p-chlorophenoxyacetic acid; picloram, 4-amino-3,5,6-trichloropicolinic acid

Introduction

Genetic transformation mediated by *Agrobacterium* or particle delivery system has become popular in modern plant breeding research. Useful genes can be introduced into plant species in an asexual way. Genetic modification may become an important tool for the improvement of

Alstroemeria, which is a monocotyledonous species. Development of an efficient plant regeneration system is a prerequisite for genetic modification (Taylor et al. 1996; Raemakers et al. 1997). In monocots, embryogenic callus has proved to be the best target for transformation (Smith and Hood 1995), mainly because the embryogenic callus provides mass-reproducible material that can increase the opportunity of transformation. In monocots, generally two types of embryogenic callus are found: compact and friable, and most of them are induced from zygotic embryos (Benmoussa et al. 1996; Bregitzer et al. 1991; Buiteveld et al.1994; Fransz and Schel 1991; Gendy et al. 1996; Jullien and Van 1994; Ke and Lee 1996; Oinam and Kothari 1995; Remotti and Löffler 1995; Teixeira et al. 1993; Yam et al. 1990). In maize, the compact (or Type I) callus has a solid appearance with many scutellum-like structures, and the friable (or Type II) callus is characterized by a soft, yellow, or white, friable appearance (Fransz and Schel 1991; Welter et al. 1995).

In *Alstroemeria*, compact callus could be induced from mature or immature zygotic embryos (Hutchinson et al. 1994; Van Schaik et al. 1996), but the friable callus was not well documented. It was only shown by Hutchinson et al. (1997) that the more 'friable' callus could be induced by preculturing compact callus on a high NAA medium for two days, and followed by a liquid culture procedure. The subsequent products were cell aggregates with a diameter between 1 and 2 mm. Except that, there is no report referring the friable callus in *Alstroemeria*.

Friable callus has proved to be a valuable source for the establishment of cell suspension cultures, which resulted in successful transformation in many crops (Kamo et al. 1990; Kamo et al. 1995; Raemakers et al. 1997; Register et al. 1994). The objectives of this study are to develop an efficient system for the initiation and maintenance of friable embryogenic callus (FEC) from stem tissues, and a system for the regeneration of plants from FEC applicable for further genetic modification research.

Materials and methods

Compact callus induction from stem segments

Self-pollinated seeds of *Alstroemeria* VV024 (a tetraploid breeding line from Van Staaveren BV, Aalsmeer, The Netherlands) and BT207 (a tetraploid 'Butterfly' type breeding line, from

Wülfinghoff Freesia BV, The Netherlands) were sterilized with 70% ethanol (1 min), 3% sodium hypochlorite solution (20 min), then rinsed 3 times with sterilized water (10 min each). After surface sterilization, the seeds were placed on medium with half-strength Murashige and Skoog (1962) salts and vitamines, 10 g/l sucrose, and solidified with 2.2 g/l gelrite, pH 5.8. Cultures were placed in 18°C, 12 h light conditions.

Two months after incubation the seeds started to germinate, and one month later, stem segments (1 cm, with one node) were excised and placed on callus induction medium. The callus induction medium consisted of Murashige and Skoog salts and vitamines, 30 g/l sucrose, 7 g/l micro agar, and 0.5-4.0 mg/l 2,4-D with or without 0.5-1.0 mg/l BAP, pH 5.8. After autoclaving, the medium was poured into 9 cm Petri dishes. Fifty explants were cultured for each treatment. All cultures were placed at 18°C room in darkness.

The explants were subcultured bi-weekly on induction medium for two months. After formation of a soft type of callus, the cultures were transferred to regeneration medium and were subcultured bi-weekly. The regeneration medium consisted of Murashige and Skoog salts, vitamines, 30 g/l sucrose, 3 g/l gelrite, and 0.5 mg/l BAP (BA0.5).

Subsequently, a compact type of callus was formed and was used as initial material for the following friable and granular callus induction experiments.

Friable and granular callus induction from compact callus

The compact calli were separated, without damaging, into single units (2-5 mm in length) by a pair of forceps, and then placed on the culture medium. Ten units were placed in a Petri dish and 5 replicates were prepared for each treatment.

I. friable callus induction

Two basal media were used in this experiment: MS and PCA. MS medium consisted of Murashige and Skoog salts and vitamines, 2% sucrose, and solidified with 3 g/l gelrite, pH 5.8. PCA medium (Sofiari et al. 1998) has the same composition as MS but is additionally supplemented with the following compounds (in mg/l): 100 myo-inositol, 18200 d-mannitol, 480 MES, 100 casein hydrolysate, 80 adeninesulphate, 0.5 d-calcium pantothenate, 0.1 choline

chloride, 2.0 nicotinic acid, 0.5 ascorbic acid, 1.0 pyridoxin-HCl, 10.0 thiamine-HCl, 0.5 folic acid, 0.05 biotin, 0.5 glycine, 0.1 L-cysteine, and 0.25 riboflavine. The basal media were supplemented with picloram (0, 1 and 10 mg/l) to induce friable callus. After seven weeks, the percentage of compact callus that had formed friable calli was calculated.

II. Granular callus induction

Media supplemented with different plant growth regulators were tested to induce granular callus. The control medium used in this experiment was BA0.5, and was supplemented with 1, 2, 4 mg/l 2,4-D (BA0.5D1, BA0.5D2, BA0.5D4, respectively). After five weeks of culture the percentage of compact callus that had formed granular calli was calculated.

Proliferation and maintenance of friable callus

MS and PCA basal media supplemented with picloram (0, 1, and 10 mg/l) were tested for proliferation and maintenance of friable calli, which were induced in the previous experiment. Approximately 300 mg of friable calli was divided into 6 equal parts and placed on the medium in a 9 cm Petri dish. Four replicates were prepared for each treatment. All cultures were kept on the same medium without refreshing for 5 weeks. The callus weight was measured after five weeks, and the proliferation rate was calculated.

All the friable calli were subcultured on the same medium for 3 weeks again. The appearance of all cultures was recorded, and the browning calli were collected and weighted in order to calculate the percentage of browning.

In order to investigate the regenerability and friability over a long subculture period, well growing calli were collected and incubated on the same medium without refreshing for another eight week. Six callus clumps of approximately 100 mg, were cultured in one 9 cm Petri dish, and 6 replicates were prepared for each tested medium. After eight weeks, the percentage of callus clumps with either embryos, shoots, roots, granular calli, or friable calli were calculated.

Somatic embryogenesis of friable callus

Friable calli, maintained on PCA medium with 10 mg/l picloram for two months, were cultured on medium supplemented with Murashige and Skoog salts and vitamines, 30 g/l sucrose, 3 g/l gelrite, and 0, 0.1, 0.5, 2.0 mg/l BAP (BA0 as control, BA0.1, BA0.5, BA2.0, respectively). Each plate (9 cm Petri dish) contained 500 mg friable calli, and four replicates were prepared for each treatment. Cultures were refreshed by a bi-weekly subculture interval.

After one month, the number of granular calli per plate was recorded. All of the granular calli formed were collected and transferred to the same fresh media. One month later, the number of somatic embryos was recorded. The percentage of embryo formation was calculated by dividing the number of embryos by the number of cultured granular calli. The browning level of the cultures was determined visually.

Regeneration of plants from somatic embryos

Somatic embryos were cultured on medium supplemented with Murashige and Skoog salts and vitamines, 30 g/l sucrose, 3 g/l gelrite, and 0.1, 0.5, 2.0 mg/l BAP, pH 5.8, for converting into plantlets. The explants were subcultured by a three-week interval. Six embryos were tested for each medium.

Results

Compact callus induction from the stem segments

After 10 weeks of culture on callus induction medium, only a few of the stem segments of both BT207 and VV024 genotypes had formed callus on the nodal region (Table 1, data only shown for genotype VV024). The callus was yellowish, with a soft, sticky texture, and continuously growing for two months.

After one month of subculture on regeneration medium (BA0.5), most of the soft calli became rhizogenic, and less than 1 % formed compact callus. In the case of the VV024, the compact callus originated from the induction medium supplemented with 4.0 mg/l 2,4-D and

1.0 mg/l BAP. In the case of BT207, the compact callus originated from the induction medium supplemented with 4.0 mg/l 2,4-D and 0.5 mg/l BAP.

Compact callus is an irregular, white or yellow colored, solid type of callus, with a smooth surface, and is longer than 5 mm in length (Fig. 1). It was strikingly different from the rhizogenic callus. The compact calli were maintained on BA0.5 medium for more than 1.5 years, by a monthly subculture interval. During this period the compact calli formed shoots and new compact calli, simultaneously.

One compact callus line, induced from genotype VV024, was selected and named VV024C, and another one, selected from genotype BT207, was named BT207C. The morphology of callus line VV024C was similar to BT207C, however, because VV024C grew more vigorous than BT207C, the following experiments were focused on VV024C.

Table 1. Callus induction on the stem segments of selfed progenies of *Alstroemeria* VV024, and 50 explants were cultured per treatment.

Medium composition (mg/l)		Soft callus formation (%)
2,4-D	BAP	
0	0	0
0.5	0.5	2.2
1.0	0.5	0
2.0	0.5	5.8
4.0	0.5	0
0.5	1.0	0
1.0	1.0	0
2.0	1.0	2.8
4.0	1.0	3.0

Friable callus induction from compact callus

Compact calli, cultured for seven weeks on basal medium (MS and PCA) without picloram, formed only shoots and roots on their marginal region. The percentage of shoot formation on MS basal medium was significantly higher than that on PCA basal medium (Table 2).

Cultured on medium supplemented with 1 mg/l picloram (MS1P, PCA1P), the compact calli produced friable calli, as well as shoots, and roots, whereas on medium supplemented with 10 mg/l picloram (MS10P, PCA10P) only the formation of friable callus was stimulated (Table 2).

Friable callus units are smaller than 0.1 mm in diameter and show a yellow, round, tiny structure (Fig. 2), and is formed as an aggregate clump on the surface of compact callus. The friable callus clumps could be separated from the surface of compact callus, and were spreaded easily on the semi-solid medium surface as a thin layer.

Table 2. Friable callus induction on VV024C compact callus. Data were collected after 7 weeks of culture. Means in the same column followed by the same letters are not significantly different at the 5% level as determined by LSD.

Medium composition			Friable callus formation (%)	Shoot formation (%)	Root formation (%)
Medium	Basal	Picloram (mg/l)			
MS	MS	0	0.0 c	70.0 a	85.0 a
MS1P	MS	1	45.0 b	5.0 c	15.0 bc
MS10P	MS	10	85.0 a	0.0 c	0.0 c
PCA	PCA	0	0.0 c	45.0 b	60.0 ab
PCA1P	PCA	1	45.0 b	10.0 c	30.0 bc
PCA10P	PCA	10	95.0 a	0.0 c	0.0 c

Granular callus induction from compact callus

Granular callus was formed on the surface of the compact callus, that was observed on all tested media after five weeks of culture. The granular callus has a yellow, round structure, with units of approximately 1 mm in diameter, larger than the friable callus units. The granular calli usually appeared in aggregates, and could be easily separated in individual units (Fig. 3). Sixty-five percent of the compact calli formed granular callus on control medium (BA0.5). The addition of 1 mg/l 2,4-D to BA0.5 medium increased the percentage of granular callus

formation, however, not significantly. Higher concentration of 2,4-D reduced the percentage significantly (Table 3).

After five weeks of culture, 42.5% of the explants turned brown on BA0.5 medium. The addition of 2,4-D in the culture media resulted in a more serious browning (Table 3). Although tissue browning was harmful and resulted in the death of the whole explant, it did not hamper the formation of granular callus. The granular callus often appeared on the surface of a dying explant.

Table 3. The formation of granular callus on VV024C compact callus using BA0.5 medium supplemented with 0, 1, 2, and 4 mg/l 2,4-D. Data were collected after five weeks of culture. Means in the same column followed by the same letters are not significantly different at the 5% level as determined by LSD.

Medium	Explants formed granular callus (%)	Explants turned brown (%)
BA0.5	65.0 ab	42.5 b
BA0.5D1	77.5 a	75.0 a
BA0.5D2	54.0 bc	70.0 a
BA0.5D4	32.0 d	82.0 a

Proliferation and maintenance of friable callus

The friable calli, separated from the surface of the compact callus, were cultured on six media for five weeks. They grew vigorously on all media tested. The proliferation rate, measured as increase of fresh weight varied from 3.6 to 4.9; however, there were no significant differences (Table 4). With the naked eye no difference was observed between calli cultured on the six different media; they were yellowish and vigorously growing. However, a binocular microscopic observation revealed that some tiny granular callus units were formed on picloram free media (MS and PCA), but not on picloram-containing media.

Browning of callus was not observed after 5 weeks of culture. After 8 weeks of culture, 0.9 - 6.3% of the friable calli turned brown. MS medium showed the highest percentage of browning, whereas MS10P medium showed the lowest (Table 4).

After eight weeks of culture on both MS and PCA media (without picloram), almost all calli were granular and visible by naked eye. On picloram-containing medium the calli remained friable (Table 4).

Incubation of the collected friable or granular callus on the same medium for eight more weeks (16 weeks in total), which resulted in significant differences among the six tested media. Considering the presence of embryo formation, the six tested media could be grouped into two categories: with (MS, MS1P, PCA, and PCA1P) and without (MS10P and PCA10P) embryogenesis. More than 50% of the granular calli developed into embryos on picloram-free medium (MS and PCA), whereas less than 5% of them formed embryos on the medium containing 1 mg/l picloram (MS1P and PCA1P) (Table 4).

The more organized granular callus was observed in all of the tested media, and the percentage ranged from 53.3% to 97.2%. Friable callus was only observed in picloram containing media, especially in the medium with 10 mg/l picloram (Table 4).

Based on the results of Table 4, PCA10P was chosen for maintenance of friable callus. The friability was maintained for more than one year by a three-week subculture interval, and with an average proliferation rate of 2.5 (data not shown).

Table 4. Friable callus proliferation of the genotype VV024C and the maintenance of the callus type after 5, 8, and 16 weeks. Means in the same column followed by the same letters are not significantly different at the 5% level as determined by LSD.

Medium composition		5 weeks [1]	8 weeks [1]		16 weeks [2]		
Medium	Picloram	Proliferation rate	Browning callus (%)	Callus appearance	Callus clumps with embryos (%)	Callus clumps with friable callus (%)	Callus clumps with granular callus (%)
MS	0	4.1 a	6.3 a	granular	66.7 a	0 b	66.7 b
MS1P	1	4.4 a	4.7 ab	friable	2.8 c	100 a	97.2 a
MS10P	10	4.6 a	0.9 b	friable	0.0 c	100 a	86.1 ab
PCA	0	4.9 a	2.6 ab	granular	55.6 b	0 b	53.3 b
PCA1P	1	3.6 a	2.0 ab	friable	2.8 c	100 a	91.7 a
PCA10P	10	4.3 a	1.8 b	friable	0.0 c	100 a	91.7 a

[1] Four replicates per treatment, each replicates contained approximately 300 mg of friable calli in the beginning.

[2] Six replicates per treatment, each replicate contained 6 callus clumps.

Somatic embryogenesis from friable callus

Media without picloram stimulated the formation of somatic embryos in a 16 week's culture (Table 4). However, the period of 16 weeks was very long. To test whether cytokinin is necessary for better stimulation, friable calli were cultured on medium supplemented with 0 to 2 mg/l BAP. During the first three weeks, the friable calli kept growing and became aggregates on all tested media, and their appearance looked similar; still yellowish and friable. After two more weeks of culture, granular calli appeared in all tested media. The number of granular callus units per plate ranged from 134.5 to 184.8; however, no significant difference was found between the tested media. A large variation was found between the plates in one treatment (Table 5; the standard deviation varied from 36.2 to 65.4).

Accompanied by growth, the callus produced phenolic compounds that resulted in browning and, subsequently, dying of the callus. This exudate had also negative effects on the surrounding calli. Starting from a small brown/black spot in a yellowish callus clump, the brown/black color extended gradually to the whole clump, and finally to the whole Petri dish. However, by frequently refreshing the culture medium and removing the brownish calli, healthy calli could be rescued. Higher concentration of BAP (2.0 mg/l) stimulated browning of the callus (Table 5).

All the granular calli, formed in the same plate, were collected and transferred to new medium for further growth. Somatic embryos (Fig. 4) were formed one month later. On a BAP free medium (BA0), 6.3% of the granular calli differentiated into embryos. On BA0.1, BA0.5, and BA2.0 medium, respectively, 6.2%, 9.3%, and 33.0% of the granular calli differentiated into embryos (Table 5). Subsequently, all the other granular calli differentiated into hard compact calli.

Table 5. The induction of embryogenesis in friable callus using 0-2 mg/l BAP in the medium.

Medium	Number of granular callus units per plate[1] (mean±S.D.)	Percentage of granular calli differentiated into embryos[2] (mean±S.D.)	Browning level[2]
BA0	134.5±57.7	6.3±4.6	+
BA0.1	183.0±65.4	6.2±1.3	+
BA0.5	135.8±36.2	9.3±4.8	+
BA2.0	184.8±55.4	33.0±13.5	++

[1] Data were collected one month after incubation: each plate contains 500 mg of friable calli, and 4 plates were prepared per treated medium.
[2] Data were collected two months after incubation.

The development of somatic embryos

To investigate the conversion procedure of somatic embryos and the subsequent regeneration to plantlets, the somatic embryos were collected from the previous experiments and cultured on medium containing 0, 0.1, 0.5 and 2.0 mg/l BAP for eight weeks. During this period, most of the somatic embryos formed granular calli on their basal parts, which developed into somatic embryos, subsequently. At the start, there were six somatic embryos cultured, and this number had increased in all media tested at the end of the experiment. The highest number of newly formed somatic embryos was obtained on BA0.1 medium (Table 6).

The somatic embryos possessed a cotyledon, a shoot apex, and a root primordium. With normal development, the shoot apex developed into a shoot and, subsequently, formed a complete plantlet with shoots and roots (Fig. 5). However, in most cases the cotyledon part became swollen and turned into non-regenerable green callus (Fig. 6). Sometimes the root primordium also developed into non-regenerable callus, but with a white color. This type of callus usually enlarged (one unit might be more than 10 g), and turned gradually brown and died. In some cases secondary somatic embryos were formed on the surface of the primary embryo, resulting in a multiple cotyledonous structure (Fig. 7).

The embryos formed shoots only on BAP-containing media, not on the BAP free medium, and BA0.5 and BA2.0 media stimulated shoot formation. Roots were observed on all media except BA2.0. Both, the formations of non-regenerable callus and of secondary embryos, were

frequently found on BA0.5 and BA2.0 medium (Table 6). The shoots, formed on MS30, BA0.1, BA0.5 medium, were normal. However, they showed abnormal growth on BA2.0 medium, with curled and dark-green leaves, multiple shoots, and fasciated stems.

Afterwards, based on the results of Table 6, plantlets with normal shoots were transferred to BA0.5 medium continulusly, with a three-week subculture interval. Two months after subculture, complete plants with rhizome, shoots, and roots were formed, which were suitable for transferring into the soil.

Table 6. Effect of 6-benzylaminopurine on the conversion of somatic embryos into plantlets.

Medium	Number of somatic embryos cultured	Number of embryos (after 8 weeks of culture)				
		in total	with shoots	with roots	with non-regenerable callus	with secondary somatic embryos
BA0	6	35	0	8	7	5
BA0.1	6	66	3	6	12	7
BA0.5	6	22	8	3	10	11
BA2.0	6	14	5	0	8	8

Discussion

The induction and formation of different types of callus

Zygotic embryos are capable of callus induction in many monocots, including *Alstroemeria* (Gonzalez-Benito and Alderson 1990; Hutchinson et al. 1994; Van Schaik et al. 1996). In only a few monocotyledonous species, full grown plant organs could be used to initiate somatic embryos. For example, in sorghum, roots and epicotyl segments have been used (Gendy et al. 1996), in asparagus spear sections (Benmoussa et al. 1996), in taro axillary buds (Yam et al.1990), in gladiolus corms (Kamo et al. 1990) and in wheat inflorescences (Redway et al. 1990). We report here for the first time the induction of callus from the stem segments of

two tetraploid *Alstroemeria* genotypes, and subsequently, the somatic embryo formation out of this callus.

In general, callus studied in monocots has either a friable or a compact morphotype. In several monocots, the composition of plant growth regulators in the culture medium directed the formation of the callus morphotype. In sorghum, medium supplemented with only 2,4-D induced friable callus formation, whereas 2,4-D together with dicamba induced compact callus formation (Gendy et al. 1996). In asparagus, pCPA together with BAP induced friable callus, and 2,4-D together with kinetin induced compact callus formation (Benmoussa et al. 1996). In taro, NAA together with BA induced friable and 2,4,5-T alone induced compact callus formation (Yam et al. 1990). In gladiolus, NAA induced compact and 2,4-D induced friable callus formation (Kamo et al.1990). In *Alstroemeria*, NAA together with kinetin and 2,4-D together with BAP induced compact callus formation from mature (Hutchinson et al. 1997) and immature zygotic embryos (Van Schaik et al. 1996), respectively. All these studies revealed that auxin was crucial for callus induction, and the addition of cytokinin was either enhancing the response or changing the direction of the response. Our results showed that 2,4-D together with BAP were necessary for the induction of callus from stem segments, and the formation of compact callus, subsequently, was accomplished on a medium supplemented with only BAP. This compact callus could be maintained for more than 1.5 years on BAP-containing medium without loosing their regeneration capability.

Subculturing the compact callus on medium supplemented with BAP, whether in combination with 2,4-D or not, induced granular callus formation (Table 3). The granular callus was able to regenerate into compact callus or into somatic embryos. On the other hand, subculture of the compact callus on picloram-containing media resulted in the formation of friable callus (Table 2). The induction and description of this friable type of callus have never been reported in *Alstroemeria* before.

The process of somatic embryogenesis and the plantlet regeneration

The process of somatic embryogenesis and regeneration in *Alstroemeria* can be summarized as shown in Fig. 8.

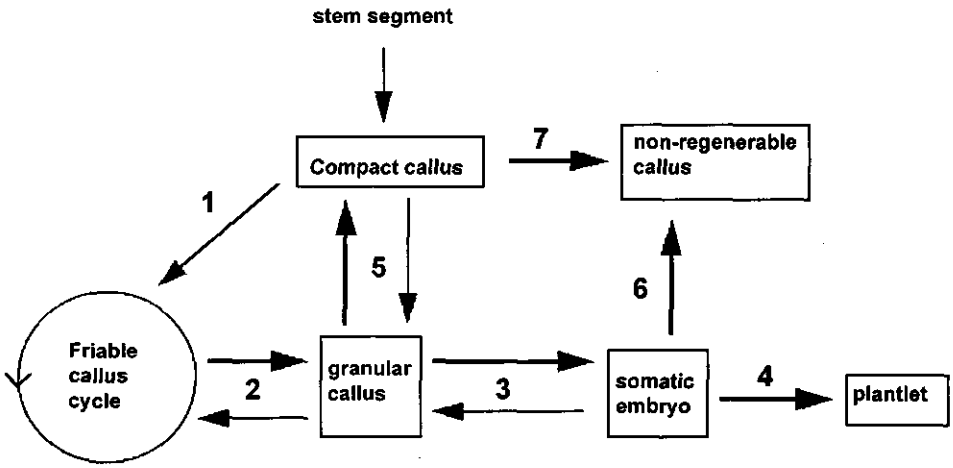

Fig. 8. Schematic representation of somatic embryogenesis in *Alstroemeria*. The arrow indicates the regeneration direction and the number represents the regeneration pathway.

Friable callus can be induced either from compact callus (pathway 1), or from granular callus (pathway 2). Proliferation and maintenance of the friable callus were achieved by using PCA medium supplemented with 10 mg/l picloram.

Granular callus is an organized tissue, which was either regenerated from friable callus (pathway 2) or from compact callus (pathway 5). Granular callus can have two different developmental pathways, either becoming an embryo (pathway 3) or becoming a compact callus (pathway 5). It was estimated that, on the commonly used regeneration medium (BA0.5), more than 90% of the granular callus became compact (pathway 5), and less than 10% developed into somatic embryos and plantlets (pathway 3 and 4).

The normal embryogenesis process is following the pathway 2-3-4, from friable callus via granular callus to somatic embryos. The formation of non-regenerable callus from cotyledon or from root primordia of the somatic embryos was named as pathway 6. The time needed from friable to granular callus was approximately 1 month; from granular callus to embryos was approximately 1 month; from embryo to a complete plant with shoots, roots, and rhizomes was approximately 4 months. So, the total time needed from friable callus to the formation of a complete plant was approximately 6 months.

The somatic embryogenesis process in *Alstroemeria*, from friable callus to embryo, was similar to that of other monocots such as Augustinegrass (Kuo et al. 1993), leek (Buiteveld et al. 1994), maize (Fransz and Schel 1991), and sorghum (Gendy et al. 1996).

In previous reports on *Alstroemeria*, suspension callus with higher friability was difficult to get and was only achieved by pre-culturing the compact callus on a high NAA concentration medium for two days (Hutchinson et al. 1997). In this report, we have shown that the compact calli were able to produce friable callus, with a high frequency, on the semi-solid picloram-containing media.

We have shown the induction, proliferation, and regeneration of friable callus in *Alstroemeria*. The friable callus can be maintained and proliferated on a single medium over a long period without loosing its embryogenesis ability. The granular callus can be produced efficiently from friable callus, and vice versa. The cyclic system of somatic embryogenesis shown in Fig. 8 provides two types of embryogenic callus, friable and granular, both can be applied in genetic modification studies.

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Fig. 1-7. Callus morphotypic differentiation and somatic embryogenesis in the tetraploid *Alstroemeria* genotype VV024C: 1) compact callus; 2) friable callus; 3) granular callus (arrows) formed on the surface of a compact callus; 4) somatic embryos; 5) plantlet regenerated from somatic embryo; 6) non-regenerable callus formed from the cotyledonous tissue of a somatic embryo; 7) secondary somatic embryos formed on the surface of a primary somatic embryo, arrow indicates the cotyledons. (bar= 0.5 mm for **Fig**. 2, 3, 6,7, 2.0 mm for **Fig**. 1, 4, and 10.0 mm for **Fig**. 5. Cm compact callus, Co cotyledon, Em embryo, Nc non-regenerable callus, R root, S shoot)


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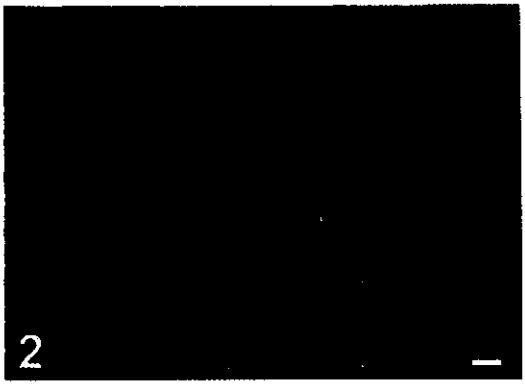
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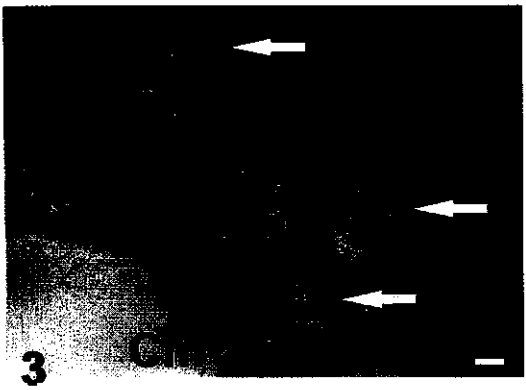
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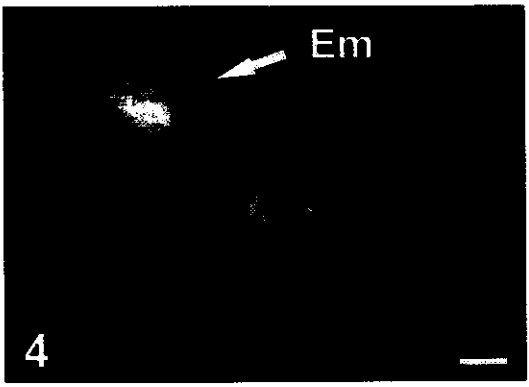
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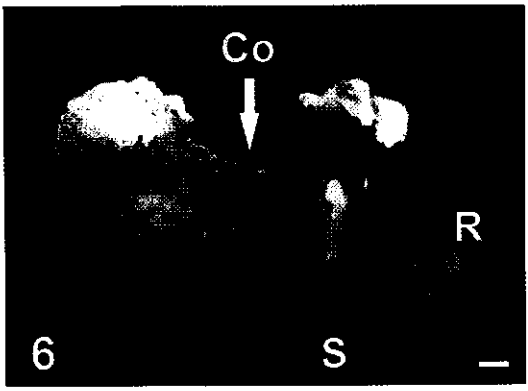
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Chapter 6

Genetic transformation of *Alstroemeria* using particle bombardment

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Abstract

Transgenic plants were obtained after particle bombardment of embryogenic callus derived from stem segments of two tetraploid *Alstroemeria* genotypes. Two plasmids containing different selection and reporter genes were used. Firstly, a plasmid containing a firefly luciferase reporter gene driven by the maize ubiquitin promoter (*Ubi1*), was bombarded into both granular and friable calli. Transient and stable expression of luciferase were detected by the luminometer after spraying the samples with luciferin solution. Visual selection assisted by the luminometer was effective. This selection is a nondestructive method, and the luciferase activity can be assayed over the whole developmental process after bombardment from callus to embryo and plantlet. Two callus morphotypes, granular and friable, tested in the experiments revealed that granular type was better than friable type for the particle bombardment mediated transformation when using luciferase as a selection marker. The second plasmid containing a selectable *bar* gene coding for phosphinotricin acetyltransferase (PAT) together with an *uid*A reporter gene coding for ß-glucuronidase (GUS). Both genes driven by the *Ubi1* promoter, were bombarded into granular callus. The transgenic calli were effectively selected from the callus clumps four months after bombardment on a medium containing 5 mg/L phosphinotricin (PPT). Selection by PPT was efficient and labor-saving. Stable expression of GUS was confirmed by the histochemical staining assay.

Key words: *Alstroemeria*, bar, GUS, PAT, luciferase, regeneration, transformation, ubiquitin promoter

Introduction

Alstroemeria is an important monocotyledonous ornamental in The Netherlands. The traditional breeding objectives were mainly focused on improving horticultural important characteristics in commercial cultivars. Interspecific crossing barriers were overcome by the use of embryo rescue techniques (Buitendijk et al. 1995; De Jeu and Jacobsen 1995); this allowing the introduction of useful genes from wild species. However, for some traits, for example virus

resistance (Bouwen and Van Der Vlugt 1996) and delayed post-harvest leaf-yellowing (Van Doorn and Woltering 1991), the necessary genes are not detected in the *Alstroemeria* gene pool yet. In this case, gene transformation mediated by *Agrobacterium* or particle delivery system is considered to be more promising, because target genes from other unrelated species can be used (Christou 1995; Jähne et al. 1995; Smith and Hood 1995; Wilmink et al. 1992).

Although some monocotyledonous plants have been transformed by *Agrobacterium tumefaciens*, the efficiency was very low and many parameters have to be improved before it becomes a routinely used technology in monocots (for review see Smith and Hood 1995). Particle bombardment, on the other hand, has been used in many more monocotyledonous species and in some of them it is now a routine technology (for review see Christou 1995; Jähne et al. 1995). In cereal crops, it was found that the choice of appropriate target tissue was of major importance (Jähne et al. 1995). Therefore, a system that provides appropriate target cells with competence for both transformation and regeneration is a prerequiste for the development of a transformation procedure (Christou 1995). Recently, a callus culture system in *Alstroemeria* was developed, producing regenerable granular and friable calli with high efficiency, that was considered to be applicable for genetic transformation (Lin et al., Chapter 5).

We report here the recovery of transgenic plants, in two tetraploid genotypes of *Alstroemeria*, after particle bombardment of granular and friable callus, using either the firefly luciferase reporter gene or the herbicide (phosphinotricin) resistance gene as a selection marker.

Material and methods

Plant material

Friable and granular calli from two tetraploid genotypes, VV024C and BT207C, were used in the following experiments. The production of friable and granular callus was described earlier (Chapter 5). Friable callus is a tiny, round shaped, aggregate, yellowish callus, of which individual units are smaller than 0.1 mm in diameter. Granular callus is a round shaped and more organized callus, in which one unit has a diameter of approximately 1.0 mm.

The friable calli were proliferated and maintained on a PCA medium (Sofiari et al. 1998) supplemented with 10 mg/l picloram (PCA10P, Chapter 5) by a three-week subculture interval. In order to induce granular callus, the friable calli were precultured on a regeneration medium, BA0.5, for eight weeks. The BA0.5 medium contained Murashige and Skoog (1962) salts and vitamines, 30 g/l sucrose, 3 g/l gelrite, and 0.5 mg/l 6-benzylaminopurine (BAP), pH5.8. Afterwards, the granular calli were collected and used for particle bombardment experiments. All the cultures were incubated in the 9 cm Petri dishes, and the Petri dishes were placed in a culture room at 18 °C and 12 h light.

Plasmid constructs

Two plasmids pAHC18 and pAHC25 (Christensen and Quail 1996), kindly provided by P. H. Quail, were used in the experiments. The plasmid pAHC18 contains the luciferase (*luc*) reporter gene (Ow et al. 1986). The plasmid pAHC25 contains the *uid*A reporter gene encoding ß-glucuronidase (GUS) and the selectable *bar* gene encoding phosphinotricin acetyl transferase (PAT). The *luc*, *uid*A and *bar* genes are all driven by the maize ubiquitin (*Ubi1*) promoter (Christensen et al. 1992, Christensen and Quail 1996).

DNA precipitation and particle bombardment protocol

Plasmid DNA was isolated and purified by using the Promega Wizard™ Megaprep DNA purification system. The final DNA concentration was 2 µg/ml in sterilized water. DNA-coating was performed by using the following procedures: 20 µg of plasmid DNA was mixed with 10 mg of gold particles (size 1.0 µm) and, subsequently, 30 µl 5M NaCl, 5 µl 2 M Tris HCl pH 8.0, 965 µl H_2O, 100 µl 0.1 M spermidine, 100 µl 25% PEG 1550, and 100 µl 2.5 M $CaCl_2$ were added. After a brief sonication (5 sec, 50 cycle/sec) and centrifugation (2 sec, 13,000 RPM), the pellet was resuspended in 10 ml 100% ethanol. For each bombardment, 160 µl of the suspended DNA-coated gold particles were pipetted and spreaded on the surface of a macrocarrier (diameter 2.5 cm, BioRad).

In order to obtain an utmost effect of bombardment, both friable and granular calli were treated in the following procedure: One week before bombardment, the most vigorously

growing calli were selected from culture medium and then transferred to a 9 cm Petri dish with fresh medium. Each Petri dish (plate) contained approximately 1.0 g of calli. One day before bombardment, all the calli were moved to the Petri dish center in a circular area of 2.5 cm in diameter. One day after bombardment, the calli were separated and spreaded again on the whole Petri dish for further growth.

The calli were bombarded with a PDS-1000/He Biolistic Particle Delivery System (BioRad, California, USA). The optimal parameters for particle bombardment, determined by pilot studies, were: 900 PSI (for granular callus) or 1100 PSI (for friable callus) gas pressure, 25 inch Hg partial vacuum, and the plant material were placed 5.5 cm below the stopping assembly.

Luciferase gene activity assays for pAHC18 transformation

Granular and friable calli from two different genotypes BT207C and VV024C were used in this experiment. Five Petri dishes of each were bombarded. The PCA10P medium was used for friable callus, and the BA0.5 medium was used for granular callus. The luciferase gene expression was assayed 1, 14, 28, and 42 days after bombardment of the granular callus, and 2, 9, 16, and 45 days after bombardment of the friable callus.

For assaying, the plant material was sprayed with 0.15 mg/l of luciferin aqueous solution, placed in a dark room and then measured by the luminometer immediately. The luminometer consists of an intensified CCD camera from Hamamatsu (Japan), with a Nikon 35 mm lens, connected to a personal computer. There are two kinds of data recorded simultaneously by this system. Qualitatively, the live plant material image and the luminescent image were taken separately by the camera and were saved as digital image files by a computer program (Argus50, Hamamatsu, Japan). Superimposition of the live image with the luminescent image revealed the responding calli with luciferase activity. Quantitatively, the amount of photons emitted by the plant material was detected, calculated, and recorded automatically by the system. After each measurement all the calli were transferred to fresh media for further growth.

GUS histochemical assays for pAHC25 transformation

GUS activity was assayed by incubating the samples in a 5-bromo-4-chloro-3-indoyl-D-glucuronic acid (X-Gluc) solution for 24 hr at 37°C as described by McCabe et al. (1988). Samples for GUS-assay were taken from one day after bombardment up till the regenerated plantlets.

Selection criteria and the regeneration of transgenic plants by using luciferase activity as a selection marker

Granular and friable calli from two genotypes BT207C and VV024C were used in these experiments. Five Petri dishes with granular callus, and 30 Petri dishes with friable callus, were transferred to fresh BA0.5 medium before bombardment. After bombardment, the calli were subcultured on BA0.5 medium continuously.

Ten days after bombardment the calli were assayed for luciferase activity and the selection procedure was started. Each luciferase positive callus clump was selected and transferred to fresh BA0.5 medium for further culture. This selection procedure was repeated on 10, 24, 58, and 72 days after bombardment. The number of positive responding callus clumps was recorded in each selection. The selection ratio (%) was determined by dividing the number of luciferase positive callus clumps with the total number of cultured clumps. Luciferase positive callus clumps, isolated 72 days after bombardment, were subcultured as individual lines. All luciferase positive callus lines were subcultured on BA0.5 medium bi-weekly in order to regenerate plants (as described in Chapter 5).

Selection criteria and the regeneration of transgenic plants by using PPT resistance as a selection marker

Granular calli of VV024C were incubated on BA0.5 medium supplemented with 0-5 mg/l phosphinotricin (PPT) to determine the optimal concentration of PPT for callus selection. Ten callus clumps per Petri dish and three Petri dishes per treatment were prepared. After four weeks of culture, the number of surviving callus clumps was recorded.

To do transformation, 10 Petri dishes with granular callus from BT207C and VV024C were refreshed onto BA0.5 medium and then bombarded with pAHC25. After bombardment, four dishes with BT207C and three dishes with VV024C callus were assayed to detect the GUS activity. Two weeks after bombardment the remaining calli were transferred to the selection medium for further culture. Afterwards, the cultures were constantly cultured on the selection medium for four months by a one-month subculture interval. In each subculture, the vigorously growing, white colored friable callus clumps were selected from the brown, black, and dying callus clumps with a surgical blade, and then transferred to fresh selection medium. Each callus clump was cultured individually. This selection procedure was repeated every month until all of the non-resistant calli died. Thereafter, each surviving callus clump was subcultured as an individual line.

Results

I. Transformation based on luciferase selection

Transient expression of luciferase in friable and granular callus

One or two days after bombardment, transient expression of the luciferase gene in bombarded calli was detected with the luminometer. The color range from blue, green, yellow, to red reflects the different levels of luciferase activity in the responding calli (Figure 1-A,1-B). Quantitative analysis showed that, both granular and friable calli of genotype VV024C, emitted a higher number of photons than the responding calli of BT207C. Independent of the genotype, the friable callus has a higher luciferase activity than the granular callus one or two days after bombardment (Figure 2).

Regardless of the genotype or the callus type, the amount of emitted photons declined drastically in about two weeks and then gradually decreased to the lowest level in about six weeks after bombardment. After 42 days, the luciferase activity in granular callus has reduced in VV024C and BT207C, respectively, to 21.7% and 28.9% of the level measured on one day after bombardment. On the other hand, 45 days after bombardment, the luciferase activity in friable

callus has reduced in VV024C and BT207C, respectively, to 0.7% and 8% of the level measured on two days after bombardment (Figure 2).

Selection and regeneration of transgenic plant expressing luciferase from bombarded granular callus

Five Petri dishes containing granular calli of both BA207C and VV024C genotypes were used for the selection of transformants. After bombardment, the granular calli were placed on the BA0.5 medium without refreshing for 10 days. During this recovering period, some brown spots were observed on the surface of the granular calli, and a brownish exudate appeared simultaneously. This exudate seemed to be toxic for the neighboring calli. Afterwards, the cultures were transferred to fresh BA0.5 medium bi-weekly.

Ten days after bombardment, 26.4% and 34.6% of the initially bombarded granular callus clumps, for genotype BT207C and VV024C respectively, were luciferase positive. These positive callus clumps were selected and subcultured on fresh media again. The second and third selection were conducted 24 and 58 days after bombardment. The percentage of luciferase positive clumps in these three selections was less than 33%. After 72 days of bombardment, the percentage was increased to 50% and 45% in BT207C and VV024C, respectively (Figure 3). Thereafter, each luciferase positive callus clump was subcultured on Petri dish as individual callus line. There were eight and 20 callus lines selected from BT207C and VV024C, respectively, and the subsequent measurement showed that all these callus clumps were luciferase positive (Table 1). On average 1.6 and 4.0 transgenic lines per bombarded Petri dish were obtained in BT207 and VV024C, respectively (Table 1). These individual calli were multiplicated on BA0.5 medium for 5 months, and the luciferase activity was stable expressed (Figure 1-C).

Granular calli, cultured on BA0.5 medium, differentiated into either somatic embryos or compact callus, or produced friable calli on their surface. Subsequently, the friable callus developed into granular callus and vice versa.

Eight months after bombardment, the calli differentiated into pro-embryos and 100% of them were luciferase positive (Figure 1-D). Somatic embryos with a bipolar structure were formed one month later, which were also completely luciferase positive (Figure 1-E,1-F).

Eleven months after bombardment, the first completely transgenic plantlet with shoot, root, and rhizome was formed from genotype VV024C (Figure 1-G, 1-H). In addition, completely transgenic plantlets were also formed from genotype BT207C (Figure 1-I,1-J).

In total, there are two transgenic lines from BT207C and 10 from VV024C yielded transgenic plants (Table 1). One year after bombardment, each transgenic line produced 5 to 20 complete plantlets, and there were more than 100 transgenic plantlets of VV024C obtained. On the other hand, only two transgenic lines of BT207C yielded plants in the same period.

Table 1. Number of luciferase positive callus lines and their derivative plantlets after particle bombardment of granular callus with pAHC18 in the two genotypes BT207C and VV024C.

Callus genotype	Number of bombarded Petri dishes	Number of luciferase positive callus lines [1]	Number of luciferase positive lines yielding plantlets [2]
BT207C	5	8	2
VV024C	5	20	10

[1] After 72 days of bombardment, luciferase expression was stable.
[2] After 11 months of bombardment, plantlets with roots, shoots, and rhizomes.

Selection and regeneration of transgenic plant expressing luciferase from bombarded friable callus

The selection of luciferase positive callus in friable callus was similar to that of granular callus. Two weeks after bombardment, individual callus units with luciferase activity were selected from the initial bombarded material, and then transferred to fresh media. This selection procedure was repeated every two week. Nine weeks after bombardment, luciferase expression stayed stable in all selected callus clumps. Thereafter, each positive callus clump was cultured individually as a trangenic callus line. In total, there were two and nine transgenic callus lines from BT207C and VV024C, respectively, were obtained (Table 2). On average 0.06 and 0.3 transgenic lines per bombarded Petri dish were obtained in the genotye BT207C and VV024C, respectively.

The friable calli cultured on BA0.5 medium, gradually turned into a granular type, and subsequently, into somatic embryos. Four to six months after bombardment, solid somatic embryos with luciferase activity were formed. Complete transgenic plants were obtained after two to four more months later. In total, there was one transgenic line from BT207C and five from VV024C yielded plantlets (Table 2).

Table 2. Summary of luciferase gene transformation by particle bombardment of friable callus in two genotypes.

Callus genotype	Number of bombarded Petri dishes	Number of luciferase positive callus lines [1]	Number of luciferase positive lines yielding plantlets [2]
BT207C	30	2	1
VV024C	30	9	5

[1] After 9 weeks of bombardment, luciferase expression was stable.
[2] After 8 months of bombardment, plantlets with shoots.

II. Transformation based on PPT selection

Optimal selective concentration of PPT

After four weeks of culture, all the tested granular calli survived on the control medium (without PPT), whereas, only 23.3% of the calli survived on the medium supplemented with 1 mg/l PPT. All of the calli was dead when cultured on the 5 mg/l PPT medium (Table 3). Therefore, BA0.5 medium supplemented with 5 mg/l PPT was used in the future experiments. Dying calli on a PPT-containing medium showed a deep brown or black color (Figure 4-A).

Table 3. Lethal dosage test of phosphinotricin (PPT) in culture medium for VV024C granular callus.

Concentration of PPT (mg/l)	Percentage of survived callus clumps (%)	Appearance of the callus
0	100.0	yellow, vigorous
1	23.3	brown, stunted
5	0.0	deep brown, dying

PPT selection and the regeneration of transformant with bar and uidA gene

One day after bombardment, 30% of the assayed granular calli contained blue spots (Figure 4-B). The number of spots per granular callus unit varied from 1 to 5.

After subculture on selection medium (BA0.5 supplemented with 5 mg/l PPT), the callus growth stopped and the callus color gradually changed to brown. The callus browning symptom caused by PPT looked different from that caused by the phenolic compounds, and the dying calli did not produce browning exudate. Two months after bombardment, almost all calli turned brown, black and were dying, however, some small white friable type of callus clumps were observed on the dying callus clumps (Figure 4-C). A GUS assay revealed that these white friable calli, which survived on the PPT-containing medium, were blue colored (Figure 4-D). From then on, each white friable callus clump was subcultured individually as a single callus line on the selection medium.

Three months after bombardment, 82 and 186 callus lines from BT207C and VV0224C, respectively, were selected. Those callus lines were subcultured on selection medium again. One month later, only 17 and 20 vigorously growing callus lines from BT207C and VV024C, respectively, were obtained (Table 4). All those callus lines differentiated into the proembryo stage, and the first embryo with a bipolar structure was formed (Figure 4-E). GUS assays revealed that the proembryogenic calli and the root tip of the first found embryo, were blue colored (Figure 4-F,G). Subsequently, all those lines grew on the selection medium vigorously. Later on, histochemical examination showed that GUS activity was only detected in 11 lines of BT207C and in 7 lines of VV024C (Table 4).

Seven months after bombardment, seven lines gave rise to plantlets in genotype VV024C (Table 4). Histochemical staining revealed that the plantlets were completely transgenic, with blue color in the whole plant tissues (Figure 4-H,I,J). On the other hand, only two transgenic lines in BT207C yielded plantlets 12 months after bombardment (Table 4).

Table 4. Summary of PAT and GUS genes transformation by particle bombardment.

Callus genotype	Number of bombarded Petri dishes	Total number of selected callus lines (PAT^+) [1]	Number of callus lines with blue staining (GUS^+) [1]	Number of transgenic lines yielding plants [2] (with both PAT^+ and GUS^+)
BT207C	6	17	11	2
VV024C	7	20	7	7

[1] Four months after bombardment, the callus was selected by medium supplemented with 5 mg/l phosphinotricin.
[2] Seven months and 12 months after bombardment for VV024C and BT207C, respectively.

Discussion

Most monocotyledonous plants are recalcitrant to *Agrobacterium* mediated transformation (for review see Jähne et al. 1995; Smith and Hood 1995). In recent years, many monocotyledonous species have been transformed by particle bombardment (Cao et al. 1992; Cabrera-Ponce et al. 1997; Christou 1997; Denchev et al. 1997; Jähne et al. 1994; Kamo et al. 1995; Kuehnle and Sugii 1992; Somers et al. 1992; Torbert et al. 1998; Vain et al. 1993; Vasil 1992; Watad et al. 1998; Wilmink et al. 1992; Xiao and Ha 1997). This paper shows that gene transformation mediated by particle bombardment is also applicable for *Alstroemeria*, a previously recalcitrant monocotyledonous species.

The most important aspect in transformation is the choice of a suitable initial explant. Primary explants, such as scutellar tissue of zygotic embryos, immature inflorescences, and microspores, have many advantages in cereal crops (Jähne et al. 1995). The main reason is that those tissues have a high regeneration capacity. Unfortunately, in most of the monocots, these kinds of tissues do not have the capacity to regenerate. Leaves, with regeneration ability, are also used as initial material for transformation. However, it often results in chimeric transformants (Denchev et al. 1997). Also in *Alstroemeria* the leaf explants have been bombarded, but this only resulted in transient expression without further plant regeneration (Lin et al. unpublished results). Therefore, embryogenic calli or suspension cells are frequently used as target explants for genetic modification in monocots. The embryogenic calli generally used in

bombardment experiments were initially induced from seeds (Cabrera-Ponce et al. 1997; Xiao and Ha 1997), immature embryos (Torbert et al. 1998), or mature plant tissues of existing cultivars (Kamo et al. 1995; Watad et al. 1998). In this report, transgenic plantlets were obtained by particle bombardment using granular or friable callus as target material. The plant regeneration process went through the somatic embryogenesis pathway, and the regenerants were all complete transgenic. From both genotypes, BT207C and VV024C, the embryogenic calli were initially induced from stem segments (Lin et al. Chapter 5), which suggests the potential application of genetic transformation in existing cultivars.

Although, at the beginning, the transient luciferase activity of friable callus was much higher than that of granular callus, at the end the number of transgenic lines recovered (per Petri dish) from friable callus, was less than that of granular callus. This is probably due to the nature of the callus. Friable callus consists of relatively small units and, therefore, the tolerance to high pressure bombardment and the recovery of injured cells might be poor. This might also be an explanation for the observations that the luciferase activity dropped so quickly within two weeks after bombardment. Plant tissue injury caused by particle bombardment has also described in other plant species like maize (Kemper et al. 1996; Vain et al. 1993).

The firefly luciferase gene appeared to be a powerful reporter gene in *Agrobacterium* mediated transformation of carrot and tobacco (Ow et al. 1986), and also in particle bombardment mediated transformation of cassava (Raemakers et al. 1997) and of *Dendrobium* (Chia et al. 1994). We report here, for the first time, the recovery of complete transgenic plants in *Alstroemeria* by particle bombardment using the luciferase activity for selection. The most important advantage of luciferase selection is the nondestructive nature of the assay (Ow et al. 1986). This approach was also demonstrated to be useful in *Alstroemeria*. The luciferase activity can be checked periodically through the whole developmental process, from callus to embryos into plantlets, without damaging the cultures.

An efficient selection system is very important for monocots transformation (Wilmink and Dons 1993). Luciferase selection has many advantages. However, the method is labor intensive and the equipments used are expensive. Furthermore, it was shown that the selection efficiency was not high enough in the beginning (Figure 3).

Selection systems based on kanamycin resistance are not generally applicable for monocots (Wilmink and Dons 1993) and therefore, the herbicide PPT was chosen in our

experiments. The plasmid pAHC25 contains the selectable *bar* gene encoding the phosphinotricin acetyl transferase (PAT), which can inactivate the herbicide PPT (Thompson et al. 1987). Taylor et al. (1993) reported transient expression of pAHC25 in six Poaceae species early after this plasmid construct was made. Recently, orchardgrass and barley were transformed with pAHC25 (Denchev et al. 1997; Koprek et al. 1996). In all those cases the herbicide bialaphos, a tripeptide form of PPT, was used as a selection agent. Our results also showed that the *Alstroemeria* can be transformed with pAHC25, and that the PPT was an efficient selection agent. The non-resistant calli could survive on selection medium for a few weeks, and thereafter they turned gradually brown and black. This reflects the nature of PPT selection. PPT inhibits the glutamine synthesis pathway causing the accumulation of ammonia in the cells, resulting in cell death (Tachibana et al. 1986a,b).

In our experiments, the PPT selection proved to be efficient and labor-saving compared to the luciferase selection. The non-resistant calli were killed by PPT and did not produce toxic fluid influencing the development of the resistant calli. Therefore, the cultures could be maintained on the selection medium for a long time without the need for subculturing. Afterwards the selection became easy because the resistant calli were white colored and the non-resistant ones were brown/black.

The confirmation of successful transformants with the PAT gene was difficult. However, the plasmid pAHC25 contained another reporter gene GUS, and the confirmation of this gene was easier. The disadvantage of this method is that PPT is an invasive agent, and that the GUS-assay represents a destructive nature.

Some transgenic lines were resistant to PPT, but were not have GUS activity. This is probable due to gene silencing (Assaad et al. 1993; Hobbs et al. 1993; Watad et al. 1998). This has to be investigated in the near future.

The two plasmid constructs used in this study contained either luciferase or PAT together with GUS gene, and all these genes were driven by the maize ubiquitin promotor (*Ubi1*)(Christensen and Quail 1996). *Ubi1* promoter was found to be more effective than other promoters in transformations of some monocots such as in Poaceae plants (Taylor et al. 1993), in maize (Christensen et al. 1992), in oil palm (Chowdhury et al. 1997), in orchard grass (Denchev et al. 1997), and in rice (Li. et al. 1997). Also in *Alstroemeria*, the *Ubi1* promoter was shown to be more effective than the other promoters (Van Schaik 1998). In our results the

activity of luciferase and GUS genes could be examined in all tested transgenic plant organs, such as roots, rhizomes, shoots, and leaves. This shows the usefulness of *Ubi1* as a promoter in *Alstroemeria* transformation.

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Figure 1. Luciferase activity in *Alstroemeria* callus, the derived embryos, and plantlets after particle bombardment with pAHC18 and selection: A) transient expression in granular callus 1 day after bombardment; B) transient expression in friable callus 2 days after bombardment; C) stable expression in selected callus clumps 5 months after bombardment; D) luminescent image of 100% transgenic pro-embryos, 8 months after bombardment (bar= 10 mm for A-D); E) live image, F) luminescent image of a transgenic somatic embryo, 9 months after bombardment (embryo length= 8.5 mm); G,I) live image, H,J) luminescent image of a transgenic plantlet, 11 months after bombardment (bar= 5 mm for G-J, A to H genotype VV024C, I and J genotype BT207C)

B
C
E
F
I
J

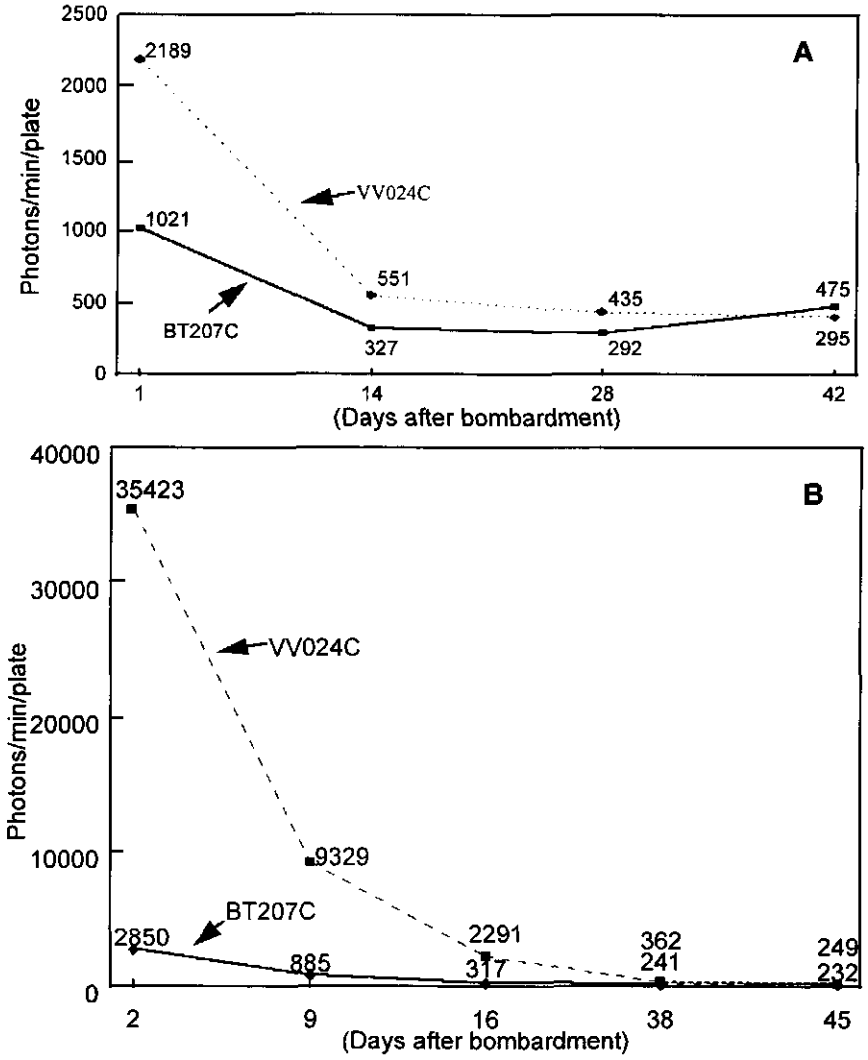


Figure 2. Transient expression of the introduced luciferase gene in two callus morphotypes of the *Alstroemeria* genotypes BT207C and VV024C after bombardment with pAHC18: A) granular callus; B) friable callus. The cultures were sprayed with luciferin solution then assayed by light emmision with the luminometer. The data were calculated on an average of 5 plates of each genotype and morphotype.

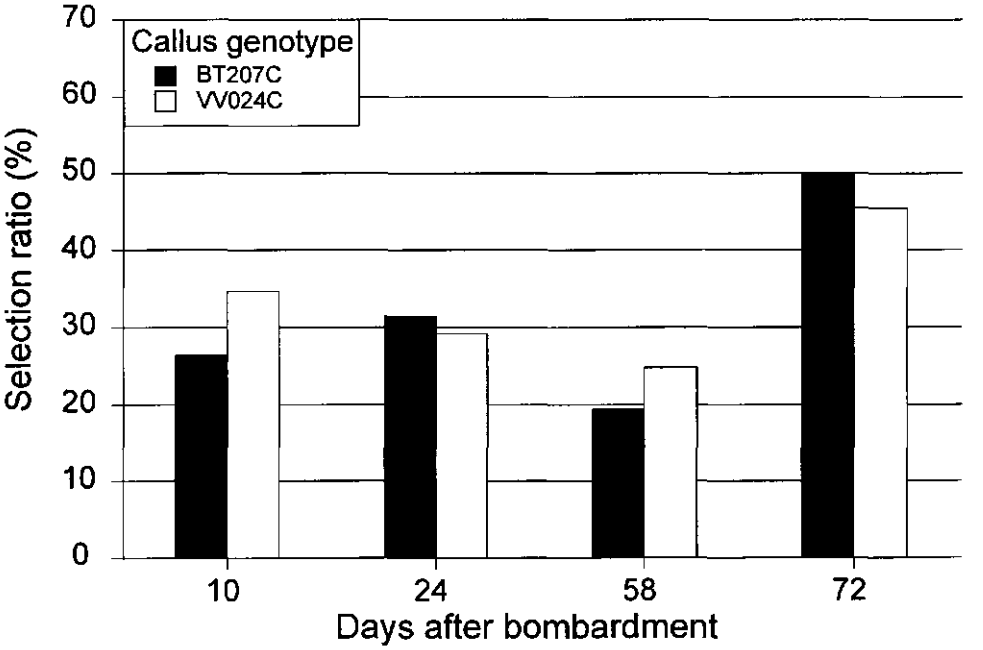
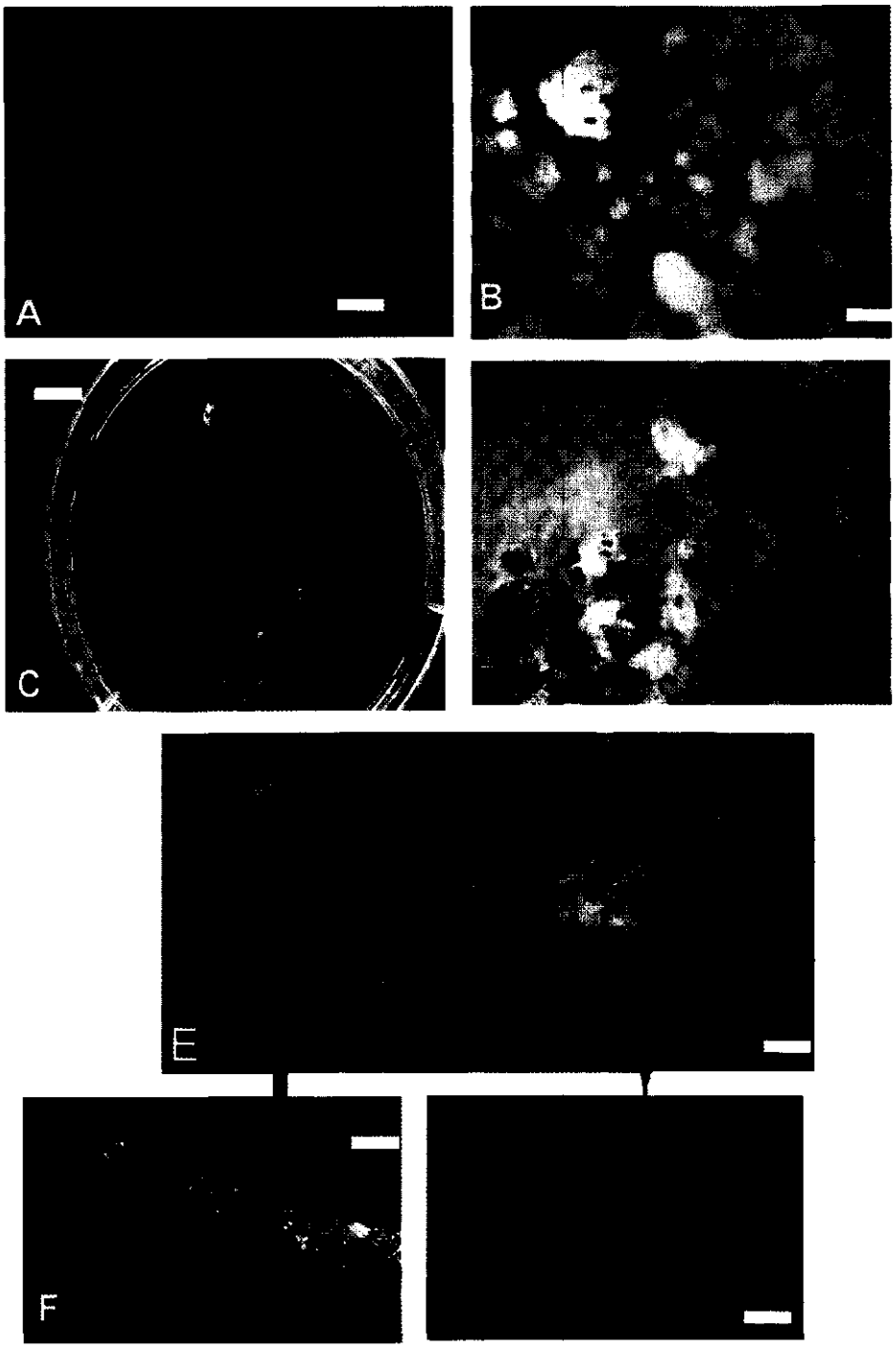


Figure 3. Selection of luciferase positive responding callus units from the VV024C granular callus clumps after bombardment with pAHC18.

Figure 4. Callus regeneration after bombardment with pAHC25. Two weeks after bombardment, the bombarded calli were cultured on phosphinotricin (PPT)-containing medium constantly. A) Non-bombarded calli dying on a medium supplemented with 5 mg/l PPT within 4 weeks (control); B) transient GUS activity in calli one day after bombardment; C) live image, D) GUS activity of white calli surviving on a PPT selection medium two months after bombardment, ; E) live image, F&G) GUS activity of embryo (root) and proembryogenic calli 4 months after bombardment; H,I,J) GUS activity in shoots, leaves and roots of several individual transgenic plants. (bar=0.5 mm for A, D, 1.0 mm for B, F, G, 5.0 mm for H, I, J, and 10.0 mm for C, E)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Chapter 7

General discussion

Regeneration of *Alstroemeria*

In this study, two different plant regeneration systems have been developed: direct organogenesis via leaf explant culture and indirect somatic embryogenesis via callus culture. Development of the leaf explant culture system is unique in *Alstroemeria* (Chapter 2). In fact, the leaf explant consists of a leaf blade and a stem node, and the new shoots regenerated directly from the leaf axils (Chapter 3). This type of regeneration pattern is generally found in dicots, because axillary buds are usually present in the leaf axils of dicots and they are able to grow. In *Alstroemeria* aerial shoots, however, no visible axillary buds were ever found in the leaf axils except at the base of the shoot near the rhizome (Buitendijk 1998). This was also confirmed by our histological observations presented in Chapter 3. The fact that shoots were initiated from epidermal cells in the leaf axil suggests an adventitious nature.

Alstroemeria was considered to be a recalcitrant species for *in vitro* culture due to its low multiplication rate, particularly in some important cultivars (Buitendijk 1998; Pierik et al. 1988). The propagation unit usually used for micropropagation is the rhizome. It was demonstrated in Chapter 4 that the leaf explant culture system is comparable to the rhizome culture system, because the plantlets derived from both systems developed into true-to-type flowering plants in the greenhouse. Besides, the leaf culture system is not only applicable for *in vitro* grown plants, but also for *in vivo* (growth chamber) grown plants and even in a commercial variety (Chapter 4). Although it took two to three months more than the conventional methods to develop a complete rhizomic plant, the disinfection of leaf material appeared to be easier and more efficient than of rhizomes. Disinfection of the underground grown rhizome tips happened to be a time-consuming work giving contamination problems that are difficult to overcome (Hakkaart and Versluijs 1988; Lin and Monette 1987; Pierik et al. 1988). In addition, the number of useful propagation units (leaves) is higher than the number of rhizomes, with a general regeneration capacity of 80% for the first three leaves.

Thidiazuron (TDZ) is a cytokinin-like substance, which is widely used to induce adventitious shoot regeneration from leaves, especially in dicotyledonous woody species (Dubois and de Vries 1995; Escalettes and Dosba 1993; Fiola et al. 1990; Huetteman and Preece 1993; Marcotrigiano et al. 1996; Turk et al. 1994). The results in this study revealed that TDZ also plays an important role in the induction of adventitious shoots in *Alstroemeria*

(Chapter 2 and 3). This substance is expected to be applicable in the conventional micropagation system as well in order to promote the multiplication rate.

Callus induction on young stem segments of two genotypes of *Alstroemeria* was described in Chapter 5. This result is comparable to other monocots such as asparagus (Li and Wolyn 1995), barley (Vitanova et al. 1995), Kentucky bluegrass (Ke and Lee 1996), *Echinochloa* (Samantaray et al. 1995), oat (Chen et al. 1995), rice (Oinam and Kothari 1995), and rye (Jia and Zhang 1993). In monocots, zygotic embryos are commonly used for callus induction, and other differentiated tissues are considered to be more difficult. In all of the above mentioned cases the calli were induced from young seedling tissues. However, there is one particular report referring that callus could be induced from bud clusters of asparagus. These bud clusters were previously induced from an excised shoot apex of mature field grown plants (Kohmura et al. 1994). In *Alstroemeria* the leaf explants were able to produce bud clusters as well (Chapter 2 and 3), which might be applicable for callus induction. More research has to be done in this field in the future.

The induction of the two callus morphotypes, friable and granular (Chapter 5), was an important progress in *Alstroemeria* research. The friable calli could be proliferated and maintained on a single solid picloram-containing medium. A liquid cell suspension culture was developed for the same purpose in *Alstroemeria* by another group (Hutchinson et al. 1997). We have also tried the liquid culture method (data not shown), but the liquid culture appeared not to be advantageous in our material; it showed to be a labor-consuming and inconvenient system, because an intensive subculture was necessary in order to prevent browning of the callus. More tools and equipments were required for the whole system, and incidental contamination happened frequently.

Granular callus is a more organized callus structure derived from friable callus on cytokinin (6-benzylaminopurine) containing medium, and it plays an intermediate role between the friable callus, compact callus, and the somatic embryo. In fact, it forms the central position of the whole regeneration system. Three different regeneration pathways can be initiated based on this type of callus. Probably this is the reason why the transformation efficiency of granular callus was higher than that of friable callus (Chapter 6).

Although the callus regeneration system has a higher multiplication efficiency than the rhizome culture system, and the true-to-type requirement may be fulfilled, the disadvantage of somaclonal variation has to be tested before it can become a routine regeneration system.

Transformation of *Alstroemeria*

The development of an efficient regeneration system is the most important factor for transformation of monocotyledonous plants. This is based on the fact that even in the most successful events, the transformation rate is still very low, and a high regeneration capability may provide more opportunities. Therefore, primary tissues with high regeneration capacity were considered to be more suitable for transformation (Jähne et al. 1995). Successful examples were shown in zygotic embryo scutellar tissues of cereal crops (Brettschneider et al. 1997; Koprek et al. 1996; Jähne et al. 1995; Takumi and Shimada 1997), leaves of orchardgrass (Denchev et al. 1997), inflorescences of sorghum (Casas et al. 1997), microspores of barley (Jähne et al. 1994), and pollen grains of maize (Horikawa et al. 1997). However, compared to dicots, the type of tissues described is very limited and may not be applicable in most of the monocots.

The leaf explant culture system provides an easy to handle plant material with high regeneration capacity (Chapter 2 and 3), which was considered to be applicable for transformation approach. We tried the particle bombardment method to deliver DNA into the regenerating leaf explants. The bombarded leaf explants were at the stage of bud primordia development: that was one week after subculture on shooting medium. Part of the leaf blade was removed before treatment in order to let the primordia tissues receive the DNA-coated particles as much as possible. Two plasmids, pAHC18 and pAHC25 (Christensen and Quail. 1996), both driven by the maize ubiguitin (*Ubi1*) promoter (Christensen et al. 1992), were used in this experiment. The pAHC18 contains a luciferase gene, and pAHC25 contains a selectable Basta resistance gene together with a GUS reporter gene. Transient luciferase and GUS activity were examined five days and four weeks after bombardment, respectively (Figure 1 and 2). The expression was observed mainly on the newly formed leaf tissues. Two months after bombardment the expression disappeared and was absent.

The transient activities expressed on the leaf tissues indicated that perhaps most of the bombarded particles only reached the L-1 layer cells of the bud primordia which differentiated into leaves subsequently. These pilot studies gave us the important information that the used DNA constructs were transferred and transiently expressed in *Alstroemeria* cells, but that selection and maintenance of transiently expressed tissue was a problem.

In the study presented, the callus shows to be superior to the leaf explant concerning the transformation efficiency. This is probably due to the fact that cells with totipotency, which could develop into a whole plant via embryogenesis, are not present in the leaves. All our successful transformants originated from somatic embryogenesis (Chapter 6), and this is in agreement with our hypothesis. The compact callus is a differentiated callus structure derived from granular callus (Chapter 5). We tried the particle bombardment method to deliver DNA into the compact callus, but we only obtained transformed chimeric structures (Figure 3 and 4). These results are comparable to those gained from the leaf explants, because the particles were mainly shot into cells without regeneration ability. It was shown that only the epidermal cells at the leaf axil region were able to regenerate into plants (Chapter 3). The compact callus is able to produce friable or granular callus, depending on the culture medium (Chapter 5). If the bombarded compact calli were cultured on a culture medium supplemented with 2,4-dichlorophenoxyacetic acid together with 6-benzylaminopurine, or on a medium supplemented with picloram, then it might be possible to get complete transgenic callus lines. However, it is still a problem to select small units from a large tissue, so that the compact callus seems not to be a good initial material for particle bombardment mediated transformation. Both granular and friable calli are capable of regeneration into plants, but the granular callus has a higher transformation efficiency than friable callus (Chapter 6).

Independent of the tested material and the DNA construct, the transient gene activity was always strong directly after bombardment, and it decreased drastically later on. This result suggests that in only a few cells, the plasmid DNA was really incorporated into the nuclear plant DNA and which could be maintained in the subsequent cell divisions. Therefore, an efficient selection method for transgenic cells seems to be very important. Either light emission or PPT selection has both advantages and disadvantages (Chapter 6), so that the production of a new DNA contruct with both luciferase and PAT genes would be helpful. The maize *Ubi1* promoter

(Christensen et al. 1992), successfully used in this study, indicates the good applicability of this promoter in the monocotyledonous genus *Alstroemeria*.

Direct plant regeneration from cultured leaves has proven to be a great potential for *Agrobacterium*-mediated transformation in many dicots such as apple (De Bondt et al. 1996), carnation (Van Altvorst et al. 1995), *Fragaria* (El Mansouri et al. 1996), and raspberry (Mathews et al. 1995). This is mainly because the explant is relatively easy to handle, and the abundant target cells in each infection may increase the probability. The single cells abundantly involved in adventitious shoot formation of the *Alstroemeria* leaf explant culture system (Chapter 3) may have the same applicability for *Agrobacterium*-mediated transformation in the future.

Alstroemeria has become a newly bred ornamental in the world in the last 20 years, thanks to the incorporation of modern breeding techniques, such as mutation breeding, *in vitro* culture, and embryo rescue. The conventional breeding efficiency based on sexual crossings and artificial mutagenesis to broaden the genetic diversity will be limited. Genetic transformation is expected to be **the** powerful tool for the transfer of useful genes in a more direct and efficient way. The successful events presented in this thesis will push the breeding activities in *Alstroemeria* forward into a luminous future.

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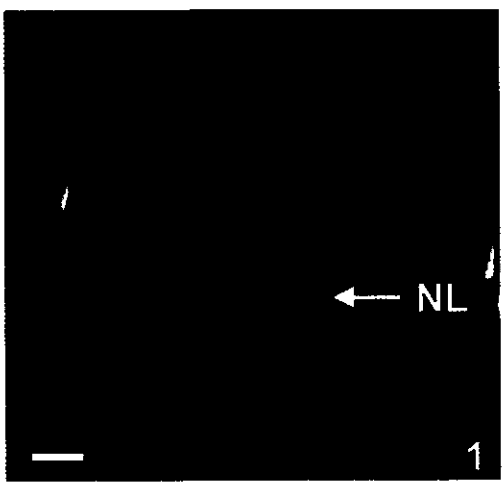
Figure 1 and 2. Transient expression of the luciferase and GUS genes in leaf tissues of *Alstroemeria* by using the leaf explants (containing a stem node) as initial bombardment targets. The leaf explants were cultured on shoot inducing medium for 10 days before bombardment in order to stimulate the cell division. **Figure 1.** The transient luciferase activity was detected 5 days after bombardment, and a newly formed leaf with luciferase activity is shown. The new elongated leaf is a part of an adventitious bud that was formed from the original leaf explant. (bar=1.0 cm) **Figure 2.** Transient expression of GUS gene was determined 4 weeks after bombardment, and the newly formed leaves showed GUS blue staining. (bar=0.2 cm)

Figure 3 and 4: Chimeric expression of luciferase and GUS genes in plant tissues of *Alstroemeria* by using the compact calli as initial bombardment targets. **Figure 3.** The luciferase activity was detected 8 months after bombardment, and it was only observed on a part of the plant tissues. A close up picture, presented at the upright corner, shows a detailed chimeric leaf. (bar=0.5 cm) **Figure 4**, the GUS activity was determined 5 months after bombardment, and only parts of the tissue showed the GUS blue staining. (bar=1.0 cm)
(Bd bud, Ca compact callus, Nd nodal region on the original leaf explant, NL newly formed leaf on adventitious buds, OL original leaf base, S stem, Sh shoot)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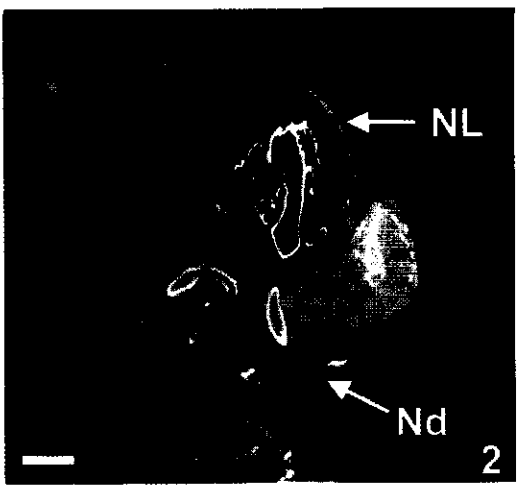
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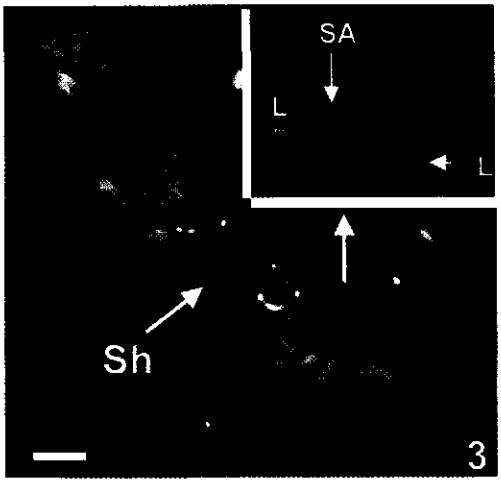
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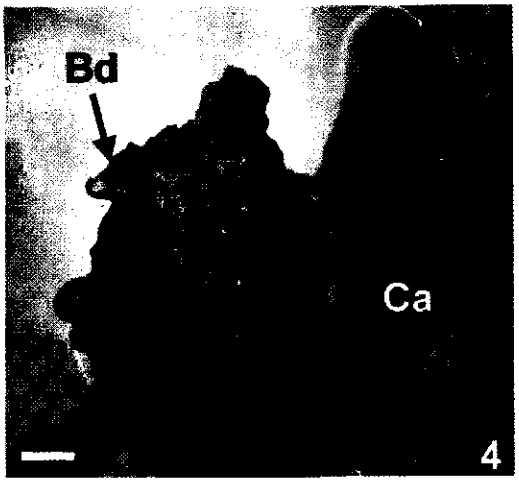
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Summary

Alstroemeria is a popular ornamental crop cultivated for its flowers. Taxonomically, it belongs to a monocotyledonous family, the Alstroemeriaceae, and is commonly called by its genus name. An *Alstroemeria* plant consists of underground grown rhizomes, roots, and aerial shoots. The plant is grown perennially. Due to the good incorporation of plant breeding techniques combined with the modern greenhouse cultivation technologies of the last two decades, *Alstroemeria* has become a competitive greenhouse-grown cut flower in the Netherlands. Generally, the *Alstroemeria* plant is vegetatively propagated by rhizome division, but the multiplication rate is rather low. Therefore, the increasing demand for plantlets stimulated the development of *in vitro* propagation techniques. However, since the multiplication unit used in the *in vitro* method is limited to rhizome tips, the propagation rate is still rather low in comparison with other crops and the other plant organs seem to be of no use during subculture. In addition, a callus culture system has been developed in the last few years for plant propagation purpose. The multiplication efficiency of this system is expected to be higher than that of the rhizome culture system, but the commercial true-to-type requirement cannot be fulfilled, because the callus was initiated from zygotic embryos. Therefore, the development of an additional *in vitro* multiplication system based on other plant organs is considered to be desirable (Chapter 1).

Plant regeneration of cultured explants has in general two pathways, either via organogenesis (the development of shoots directly on an explant) or via embryogenesis (the development of differentiated somatic embryos on an explant). A large part of this thesis research deals with the development of two plant regeneration systems, one based on organogenesis and the other based on embryogenesis. In Chapter 2, a two-step protocol for the induction of shoot formation from *in vitro* grown *Alstroemeria* leaf explants is described. Leaf explants were cut from seedlings still containing a leaf blade and a stem node. After 10 days of culture on an induction medium, the leaf explants were transferred to a shooting medium for eight weeks. New shoots were formed directly from the area adjacent to the region between leaf

base and node tissue within three weeks on shooting medium. It was histologically demonstrated that these shoots were initiated from the epidermal cells at leaf axils (Chapter 3). There were no pre-existing axillary buds ever found on the aerial leaf axils, so that this kind of organogenesis suggests an adventitious nature. The leaf explants together with newly formed shoots were subcultured several times and many normal plantlets with rhizomes were formed, which then were suitable for transferring to the soil (Chapter 2, Chapter 4).

The best induction was obtained on a Murashige and Skoog's (1962) medium supplemented with 10 μM thidiazuron (TDZ) and 0.5 μM indole butyric acid (IBA) (Chapter 2). The shooting medium contained MS medium with 2.2 μM 6-benzylaminopurine (BAP). The shoot regeneration capacity of the excised leaf explants was related to the position of the leaf on the stem. The youngest explant which was located the nearest to the shoot apex, gave the highest response. A lower gradient response was found in the leaf explants derived from leaves cut off at a further distance from the apex. This was measured in percentage of shoot regeneration per leaf explant and in the number of shoots per regenerating explant (Chapter 3).

A demonstration experiment was carried out in the greenhouse in order to investigate the similarity of plant growth morphology in between plants, which were obtained from either rhizome multiplication or leaf explant culture system. The plants were grown in the greenhouse to flowering, and the results indicated that plants obtained from both systems were morphologically identical (Chapter 4). This implicates that the leaf culture system seems to be a reliable *in vitro* propagation technique for the genotype we have investigated.

Another advantage of the leaf explant culture system is that the leaf explants directly can be excised from *in vivo* full grown shoots, and that the disinfection of aerial shoots is easier than that of underground grown rhizomes (Chapter 4). Therefore, this technique is suitable for the initiation of *in vitro* propagation of existing cultivars.

In the conventional micropropagation system, only the rhizome tips are multiplied and therefore, the aerial shoots are always discarded during subculture. In this thesis research, it is concluded that not only the rhizome tips can be used as propagation units, but also the discarded shoots can be used for the initiation of the other propagation system. The first three leaves excised from each shoot have an average regeneration capacity of 87.7%, and the average number of newly formed shoots per explant was 5.3 (Chapter 4). On the other hand, the

rhizomes can be multiplied simultaneously. Therefore, combining the rhizome multiplication system with the leaf explant culture system, the multiplication efficiency will be enhanced.

In Chapter 5, a somatic embryogenic callus regeneration system is described. A soft and sticky type of callus was induced initially from the stem segments of one month old seedlings of two tetraploid *Alstroemeria* genotypes. The soft calli turned into compact type after subculture on a medium (MS with 30 g/l sucrose) containing 6-benzylaminopurine. Subsequently, two other different morphotypes of callus, friable and granular, were obtained by subculturing the compact callus on different culture media. The friable callus can be maintained on a single medium (PCA) containing 10 mg/l picloram for a long period without loosing its friability. Subculturing the friable callus on plant growth regulator free media or on 6-benzylaminopurine containing media stimulated the granular callus formation, and the subsequent somatic embryogenesis. The somatic embryos were able to develop into complete plants.

The granular callus proved to be an intermediate between friable callus, somatic embryo, and compact callus. The friable callus could also be induced from granular callus, and vice versa. Therefore, a cyclic reproduction system was established in this research. This system provides two types of callus with a high embryogenic capability, which were initially derived from the stem segments. Thus, this system is considered to be applicable for the *in vitro* propagation of *Alstroemeria*.

In addition to the purpose of plant propagation, the development of a plant regeneration system is also considered to have the potential for genetic modification in *Alstroemeria*. Some characteristics, for example virus resistances, are very important in the continuously greenhouse-grown cultivars. However, virus resistance genes are not generally present in the *Alstroemeria* gene pool yet, so that the traditional breeding techniques are not sufficient for this purpose. Genetic transformation of *Alstroemeria* is considered to be useful for breeding in the future (Chapter 1). For genetic transformation, four important factors should be taken into account: a) an efficient DNA delivery system, b) the appropriate target cells competent for both transformation and regeneration, c) an adequate promoter, and d) a good selection system (Chapter 1).

In this research, the particle bombardment delivery system was chosen for the monocot *Alstroemeria*, because of its expectedly higher transformation efficiency than the *Agrobacterium* vector system (Chapter 1). The leaf explant regeneration system was tested for

gene transformation by using the particle bombardment. Although the gene expression could be detected after particle bombardment, the gene activities were only transiently expressed on leaf tissues, and they disappeared within two months (Chapter 7). On the other hand, the somatic embryogenic callus regeneration system was successfully used for particle bombardment mediated gene transformation. Two tetraploid *Alstroemeria* genotypes were transformed, and many transgenic plants were obtained (Chapter 6).

Both granular and friable calli were used as bombardment targets, and the subsequent somatic embryogenesis resulted in the formation of complete transgenic plantlets. Two plasmids containing different selection and reporter genes were used. Firstly, a plasmid containing a firefly luciferase reporter gene, driven by the maize ubiquitin promoter (*Ubi1*), was bombarded into both granular and friable calli. The luciferase activity was measured by a luminometer after spraying the bombarded plant material with a luciferin solution. Visual selection of the luciferase positive calli, assisted by the luminometer, was effective. This kind of selection has a nondestructive nature, without injuring the plant material, and the luciferase activity can be assayed periodically over the whole developmental process from callus to embryo and plantlet. It was shown that the granular callus is more suitable for particle bombardment mediated transformation using luciferase activity as selection marker than the friable callus (Chapter 6).

Secondly, another plasmid containing the selectable Basta (herbicide) resistance gene (*bar*) encoding phosphinotricin acetyltransferase (PAT) together with an *uid*A reporter gene encoding ß-glucuronidase (GUS) was used. Both genes were driven by the *Ubi1* promoter. The granular calli were bombarded in this experiment. Selection of the phosphinotricin (PPT) resistant calli was accomplished by culturing the bombarded calli on a medium containing 5 mg/l PPT. The PPT resistant calli were the friable type of calli which were already regenerated from the granular calli, and they developed into somatic embryos, and subsequently into the plantlets. Stable expression of the GUS gene was confirmed by histochemical staining. The blue color was detectable in all tissues of the transgenic plants tested by the GUS assay. The PPT selection proved to be a more efficient and labor-saving method compared to the luciferase selection (Chapter 6).

The results described in this thesis are beneficial for both the *in vitro* propagation and the genetic modification of *Alstroemeria*. The use of leaf explants as *in vitro* propagation units

is rather unique in *Alstroemeria*, which opens an alternative way for enhancing the plant propagation efficiency. The embryogenic callus regeneration system described in this thesis is not only applicable for plant propagation, but also for genetic transformation. The establishment of particle bombardment mediated transformation techniques will push the molecular breeding in *Alstroemeria* forward into a luminous future.

H. S. Lin. 1998 . De ontwikkeling van twee " *in vitro* vermeerderingssystemen", één via bladexplantaten en de ander via callus cultuur en de toepassing van deze vermeerderingssystemen voor genetische transformatie in *Alstroemeria.*

Samenvatting

Alstroemeria is een populair siergewas dat geteeld wordt voor de productie van snijbloemen. Het gewas wordt taxonomisch ingedeeld bij de monocotyle Alstroemeriaceae en de snijbloem wordt genoemd naar de geslachtsnaam. Een *Alstroemeria* plant bestaat uit ondergronds groeiende rhizomen, wortels en bovengrondse scheuten. De plant is meerjarig. Dankzij de toepassing van plantenveredelingstechnieken, gecombineerd met de meest moderne teeltmethoden onder glas van de laatste twee decennia, is *Alstroemeria* uitgegroeid tot één van de belangrijkste snijbloemen onder glas in Nederland. In het algemeen wordt de plant vegetatief vermeerderd door scheuren van het rhizoom; de vermeerderingsfactor bij deze vermeerderingswijze is echter laag. De immer groeiende vraag naar planten heeft de ontwikkeling van "*in vitro* vermeerderingstechnieken" gestimuleerd. Aangezien de "*in vitro* vermeerderingstechniek" beperkt is tot de vermeerdering van rhizomen, blijft de vermeerderingsfactor relatief laag vergeleken met die van andere gewassen. Andere plantendelen blijken niet geschikt voor de vermeerdering. Met het oog op vermeerdering is de laatste jaren een calluscultuur ontwikkeld, waarvan wordt verwacht dat de vermeerderingsfactor hoger is dan die van de rhizoomtechniek. De op deze wijze geregenereerde plantjes zijn echter niet identiek aan de oorspronkelijk moederplant, aangezien uitgegaan is van bevruchte embryo's bij de ontwikkeling van het callus. De ontwikkeling van een "*in vitro* vermeerderingssysteem"gebaseerd op vegetatieve plantorganen wordt beschouwd als een belangrijke stap in de verbetering van de vermeerderingsmethode (Hoofdstuk 1).

De regeneratie van planten geschiedt in het algemeen via twee processen: via organogenese (directe ontwikkeling van scheuten op een explantaat) of via embryogenese (de ontwikkeling van gedifferentieerde somatische embryo's op een explantaat). Een groot gedeelte van dit proefschrift handelt over de ontwikkeling van twee vermeerderingssystemen in *Alstroemeria,* de één gebaseerd op organogenese en de ander op embryogenese. In Hoofdstuk 2 wordt een twee-stappen protocol beschreven voor de ontwikkeling van scheutjes uit *in vitro* gegroeide bladexplantaten. De bladexplantaten, compleet met bladschijf , bladvoet

en stengelknoop, werden gesneden van *in vitro* gekiemde zaailingen. Na tien dagen opkweek op een inductiemedium werden de explantaten overgebracht op een scheutontwikkelingsmedium voor een verdere opkweekperiode van acht weken. De nieuwe scheuten werden binnen drie weken op het scheutontwikkelingsmedium gevormd, direct op het weefsel tussen bladbasis en stengelknoop. Op grond van een histologische studie van dit ontwikkelingsproces kwam aan het licht dat deze scheuten ontstaan waren uit de epidermiscellen in de bladoksels (Hoofdstuk 3). Er zijn geen rudimentaire okselknoppen in de bladoksels van opgaande scheuten aangetroffen tijdens deze studie. Derhalve moet deze vorm van organogenese als bijkomende knopvorming worden beschouwd. De bladexplantaten werden tegelijk met de nieuw gevormde scheuten enkele malen overgezet op een vers scheutontwikkelingsmedium. Daarna werden normale planten met rhizomen gevormd, die geschikt waren voor verdere opkweek in de grond (Hoofdstuk 2, Hoofdstuk 4).

Het beste scheutinductiemedium bleek het MS medium (met 30 g/l sucrose) te zijn waaraan 10 µM thidiazuron (TDZ) en 0.5 µM indole butyric zuur (IBA) was toegevoegd. Het scheutontwikkelingsmedium was MS medium met 2.2 µM 6-benzylaminopurine (BAP,Hoofdstuk 2). Het scheutregenererend vermogen van de afgesneden bladexplantaten bleek gerelateerd te zijn aan de positie van het blad op de scheut. Het jongste bladexplantaat, dat het dichtst bij het scheutmeristeem was gelegen, gaf het hoogste percentage scheutontwikkeling. Het scheutregenererend vermogen nam gradueel af in de oudere bladexplantaten die verder van het topmeristeem gesneden waren. Dit regenererend vermogen werd uitgedrukt in het percentage scheutregeneratie per blad en in het aantal scheuten per regenererend bladexplantaat (Hoofdstuk 3).

In de kas is een demonstratieproject uitgevoerd om te onderzoeken of de plantmorfologie van planten die via rhizome vermeerdering waren vermeerderd gelijk was aan die van planten die via bladexplantaten waren vermeerderd. De planten in de kas zijn tot bloei opgekweekt. Uit de gegevens over de planthabitus en de bloemmorfologie bleek dat de planten uit beide vermeerderingssystemen morfologisch identiek waren (Hoofdstuk 4). Hieruit mag worden geconcludeerd dat de vermeerderingswijze via de bladexplantaten een betrouwbare *in vitro* vermeerderingswijze is voor de in dit onderzoek geteste genotypes.

Een ander voordeel van de vermeerdering via bladexplantaten is, dat bladexplantaten direct van scheuten van volwassen planten *in vivo* gesneden kunnen worden. Het

desinfecteren van bovengrondse scheuten is daarbij gemakkelijker dan dat van ondergrondse rhizomen (Hoofdstuk 4). Deze methode lijkt dan ook zeer geschikt voor het opstarten van *in vitro* vermeerdering van bestaande cultivars.

In de gangbare vermeerderingsmethode worden alleen rhizomen vermeerderd door versnijden waarbij de opgaande scheuten steeds afgesneden en weggegooid worden tijdens de vermeerderings-stappen. Uit dit onderzoek is gebleken dat niet alleen de rhizomen kunnen worden gebruikt voor de vermeerdering van *Alstroemeria*, maar dat ook de afgesneden scheuten kunnen worden gebruikt voor de opstart van een ander type vermeerdering. De eerste drie bladexplantaten van elke scheut hebben een gemiddeld scheutontwikkelingsvermogen van 87.7 % en het gemiddeld aantal nieuw gevormde scheuten per explantaat is 5.3 (Hoofdstuk 4). Tegelijkertijd kunnen de rhizomen vermeerderd worden zodat in combinatie met de bladexplantaatmethode de vermeerderingsfactor kan worden vergroot.

In Hoofdstuk 5 wordt een callus-systeem beschreven waarbij plantjes via somatische embryogenese worden geregenereerd. Een zacht en kleverig type callus was geïnduceerd uit stengelstukjes van zaailingen van twee tetraploide *Alstroemeria* genotypen. Dit zachte callus veranderde in een compact type callus na herhaalde opkweek op een MS medium (met 30 g/l sucrose), verrijkt met 2.2 µM 6-benzylaminopurine. Vervolgens werden twee andere typen callus, het 'losse' en het 'korrelige' type, verkregen door herhaalde opkweek van het compacte callus op verschillende kweekmedia. Het 'losse' type callus kan voor een langere tijd gekweekt worden op een PCA medium met 10 mg/l picloram zonder dat het de 'losse' structuur verliest. Herhaaldelijk kweken van dit 'losse' type callus op media zonder plantengroeihormonen of op media met 6-benzylaminopurine, stimuleerde de vorming van het 'korrelige' type callus dat vervolgens somatische embryo's ging vormen. Deze somatische embryo's ontwikkelden zich verder tot complete plantjes.

Het 'korrelige' type callus bleek een overgangsvorm te zijn tussen (1) het 'losse' type callus, (2) callus dat somatische embryo's vormde en (3) het compacte type callus. Het 'losse' type kon ook ontwikkeld worden uit het 'korrelige' type en vice versa. Op deze wijze is een cyclisch reproductiesysteem ontwikkeld, waarin oorspronkelijk twee typen callus uit stengelstukjes zijn ontwikkeld, die beide een goed embryovormend vermogen hebben. Hieruit kunnen we concluderen dat dit systeem geschikt is voor de *in vitro* vermeerdering van

Alstroemeria.

Het ontwikkelen van een plantenreproductiesysteem wordt naast het belang voor vermeerdering ook gezien als een belangrijke voorwaarde voor de ontwikkeling van een genetische modificatiemethode in *Alstroemeria.* Sommige eigenschappen zoals bijvoorbeeld virusresistentie zijn zeer belangrijk in de meerjarige teelt van cultivars onder glas. De aanwezige virusresistentiegenen zijn echter nog niet getraceerd in de *Alstroemeria* species zodat de klassieke wijze van veredeling nog niet kan worden toegepast voor dit doel. De ontwikkeling van een transformatiemethode wordt dan ook gezien als een goede investering voor de toekomstige veredeling (Hoofdstuk 1).

Er zijn vier belangrijke factoren noodzakelijk voor de ontwikkeling van een succesvolle transformatiemethode:

a) een efficiënt gen-DNA overdracht systeem

b) de juiste cellen die geraakt moeten worden en die zowel kunnen transformeren als kunnen regenereren

c) een geschikte promoter

d) een goed selectiesysteem (Hoofdstuk 1).

In dit onderzoek is gekozen voor het schieten met kleine kogeltjes gecoat met DNA, omdat verwacht werd, dat dit een betere transformatie efficiëntie zou opleveren in het monocotyle gewas *Alstroemeria* dan het *Agrobacterium*-vectorsysteem (Hoofdstuk 1). Het bladexplantaat-regeneratiesysteem is getest in de transformatie methode via beschieten met DNA-gecoate kogeltjes. Hoewel genexpressie na beschieting is gevonden, bleek dit alleen van voorbijgaande aard te zijn en beperkt tot plekken in het bladweefsel. Het was na twee maanden verdwenen (Hoofdstuk 7). Het somatische embryogene callus-systeem is eveneens getest via beschieten met DNA gecoate kogeltjes. Dit bleek succes te hebben. Twee tetraploïde *Alstroemeria* genotypen zijn op de volgende wijze getransformeerd. Met deze methode zijn veel transgene planten verkregen (Hoofdstuk 6).

Zowel het 'korrelige' als het 'losse' type callus werden beschoten en vervolgens geregenereerd via embryovorming tot volledig transgene planten. Voor de transformatie zijn twee plasmiden gebruikt die verschillende selectie- en reportergenen bevatten. In een eerste experiment werden kogeltjes gecoat met een plasmide, dat het luciferasegen, afkomstig uit het vuurvliegje, aangedreven door de ubiquitine promoter uit mais (*Ubi1*) bevatte, geschoten

in zowel 'korrelig' als het 'losse' type callus. De luciferase-activiteit is gemeten met behulp van een luminometer nadat het beschoten plantmateriaal was bespoten met een luciferine oplossing. Selectie op het oog van de lichtgevende calli, ondersteund door de luminometer, bleek effectief te zijn. Deze vorm van selectie is niet destructief en kan zonder beschadiging van plantmateriaal worden toegepast tijdens het gehele ontwikkelingsproces van callus via embryo tot plant. Het 'korrelige' type callus bleek een beter uitgangsmateriaal voor transformatie met het luciferasegen te zijn dan het 'losse' type callus (Hoofdstuk 6).

In het tweede experiment werd een plasmide met het Basta (herbicide) resistentiegen dat codeert voor phosphinotricin acetyltransferase (PAT) en het *uidA* reportergen coderend voor het ß-gluceronidase (GUS), gebruikt. Beide genen werden aangedreven door de *Ubi1* promoter. Dit plasmide werd gebruikt bij de beschieting van alleen het 'korrelige' type callus. De selectie van phosphinotricin (PPT) resistente calli werd uitgevoerd door de beschoten callusklompjes te kweken op medium met 5 mg/l PPT. De PPT resistente calli bleken te bestaan uit het 'losse' type callus dat ontstaan was bovenop het 'korrelige' type callus. Deze resistente calli ontwikkelden zich via embryo's tot plantjes. In deze plantjes werd de stabiele expressie van het GUS-gen aangetroffen na chemische kleuring van het weefsel. Het was aantoonbaar in de vorm van blauwkleuring van alle weefsels van de transgene *in vitro* planten. De PPT selectie bleek een efficiëntere en minder arbeidsintensieve methode te zijn, vergeleken met de visuele selectie via het luciferasegen (Hoofdstuk 6).

De resultaten van de experimenten in dit proefschrift zijn veelbelovend zowel voor de "*in vitro* vermeerderingswijze" als voor de genetische modificatie van *Alstroemeria.* Het gebruik van bladexplantaten voor de "*in vitro* vermeerderingswijze" is uniek voor *Alstroemeria* en biedt perspectief voor de verbetering van de vermeerderingsfactor. Het somatische embryogene callus-systeem bleek niet alleen toepasbaar voor vermeerdering maar ook voor genetische transformatie. Het feit dat nu een efficiënte transformatiemethode beschikbaar is, zal het pad van de moleculaire veredeling in *Alstroemeria* verlichten tot ver in de 21 ste eeuw.

摘 要

百合水仙 (Alstroemeria) 爲近年來廣受歡迎的一個花卉作物，在植物學上是屬於阿爾氏科 (Alstroemeriaceae) 的單子葉植物，原生於南美洲智利、巴西一帶，其英文普通名通常是直接以其屬名稱之，在文獻報導上亦有稱之爲祕魯百合、印加百合者。一個百合水仙植株包括有：地下橫生的根莖、根、以及向上生長的莖枝條，爲一多年生植物。過去二十多年來，由於善用現代育種科技與結合溫室栽培技術，使得這種原生於南美洲的植物成爲荷蘭境內非常具有競爭力的溫室栽培切花。百合水仙通常用無性分生法來繁殖，以其根莖莖頂爲主要繁殖器官，唯其繁殖率很低。隨著種苗需求量的大增，組織培養繁殖法乃應運而生。由於組織培養繁殖法還是仰賴根莖莖頂爲唯一的繁殖體，其他植物器官則毫無用處，因此其繁殖效率與其他作物相比仍舊是太低。除此而外，近年來發展出來的癒傷組織培養法，其繁殖效率比較高，但是那些癒傷組織只能由雜交胚誘導產生而來，而非從既有的品種而來，也就無法滿足商業上種苗必須型實相符的要求。因此，發展出一套組織培養系統，可以利用其他植物器官作爲繁殖體以增進其繁殖效率，是一個值得考慮的方向。

組織培養時，植物再生一般經是由兩種途徑達成，一爲器官發生，植物枝條直接由培植體上產生；另一爲體胚發生，培植體透過產生體胚的方式以產生新植物。本研究之一大部份即用在建立百合水仙的這兩種植物再生體系。本論文第二章敘述一種兩階段式的葉培植體培養法的發展過程，葉培植體是由試管中生長的實生幼年植株的莖上取來，每一個培植體包含有一個葉片，及一個莖節。葉培植體先放置在誘導培養基上十天，之後換到發育培養基上培養八週。當換到發育培養基三週時，新的植物枝條即直接在葉培植體上長出，其萌發的位置介於葉片及莖節之間。利用組織切片法研究証實，這些新生枝條是由葉腋部份的莖上表皮細胞發育而來，由於切片並未觀察到葉腋有潛伏的腋芽存在，因此這些枝條是一種經由器官發生途徑產生的不定芽而來（第三章）。葉培植體伴隨著新生枝條，經過多次的繼代培養之後，變成多個具有根莖器官的正常植株，可以移植到土中（第二章及第四章）。

最佳的枝條誘導培養結果是在一個含有10 μM thidiazuron (TDZ)加 0.5 μM引朵丁酸（IBA）的MS培養基上所得到（第二章）。枝條發育用之培養基則爲MS加2.2 μM 6-benzylaminopurine(BAP)。葉培植體再生新生枝條的能力與其在莖上生長的位置有關，越年輕（即越接近莖頂）者，其再生能力越強，且其產生的芽體數越多，反之則越弱、越少，而呈現出一種向極性梯階式表現的現象（第三章）。

爲了比較不同繁殖來源的植物之生長習性，將利用傳統組織培養法，以根莖頂芽分芽培養而來的植株，與由葉培植體繁殖而來的植株，一同種在溫室中觀察，直至開

花期爲止。其結果顯示，由兩種繁殖法得到的植株，其形態學上的外觀完全一樣（第四章）。本項試驗証明葉培植體培養系統與傳統組織繁殖法並無二致，可以爲百合水仙大量繁殖所採行。

除此之外，這種培養系統還有一個優點是，所用的葉培植體不但可以取自試管生長的幼年植株，還可以直接取自溫室中生長的成年植株，而且其消毒的程序較爲簡易而安全，不似消毒地下根莖那般困難（第四章）。因此這種繁殖法極適合於現有品種之繁殖。

在傳統的組織培養系統中，只有根莖莖頂才可能增殖，其他器官都不可能，因此莖枝條在培養過程中都是予以切除棄去。本研究顯示，不僅根莖莖頂可用於繁殖，葉培植體也可做爲繁殖的材料。每一個枝條最頂端的三個葉片，平均有87.7%的再生能力，而每一個培植體平均可以產生5.3個枝條，之後這些枝條可以轉變成植株（第四章）。因此，結合根莖莖頂培養法以及葉培植體培養法，可以有效的增進百合水仙的繁殖率。

本論文第五章敘述另一種體胚化癒傷組織再生植株的培養體系。最初選用兩個百合水仙因子型之自交幼苗進行試驗，取一個月齡之幼株的莖，切段後再行培養，而得到一種軟而黏的癒傷組織，參試的二個因子型均可獲得。將這種軟的癒傷組織移到含有6-benzylaminopurine的培養基上培養後，得到另一種硬塊狀的緊密型(compact)癒傷組織。再將緊密型癒傷組織移到其他含有不同成份的植物生長調節劑的培養基上培養，結果獲得另外二種不同型的癒傷組織：分別是顆粒型(granular)及粉末型(friable)。粉末型癒傷組織可在一種含有單一生長調節劑picloram(10mg/L)的培養基上進行增殖，並保存一段長時間，而不失去其粉末狀的特性。將粉末型癒傷組織放在不含植物生長調節劑、或放在含有6-benzylaminopurine的培養基上培養，可促使其產生顆粒型癒傷組織，接著產生體胚分化現象。體胚最後會形成完整植株。

顆粒型癒傷組織據研究証明是一種介於粉末型、緊密型癒傷組織、和體胚的中間物，而粉末型也可以由顆粒型誘導而得，反之亦然，因此而形成一種循環式生產體系。這種循環體系可產生二種具有高度體胚分化能力的癒傷組織，其最初是由莖切段誘導而來，不是由雜交胚而來，因之適於百合水仙微體繁殖之用。

除了植物繁殖目的之外，發展出來的這二種植物再生體系，也可能應用於遺傳改造方面的研究。某些植物遺傳性狀，例如抗病毒病基因，對於長期栽培於溫室中的百合水仙栽培品種來說非常重要，但是這種基因至目前爲止並未在百合水仙的基因庫中發現，因此應用傳統育種技術並無法解決這類問題，而未來可能要藉助基因轉殖(transformation)的技術來達成(第一章)。要獲得一項成功的基因轉殖必須先行考慮下列四項重要因素：一）有效的去氧核糖核酸(DNA)傳送系統，二）合適的細胞，其必須具備有忍受轉殖處理的耐力，並且在處理後還有再生能力，三）合適的基因啓動

子，四）好的選拔體系（第一章）。

本研究選擇粒子鎗作爲百合水仙基因轉殖試驗用的DNA傳送系統，乃由於粒子鎗在其他許多單子葉植物轉殖試驗中具有較高的轉殖效率(第一章)。本研究曾利用粒子鎗將基因打進去葉培植體，結果顯示送進去的基因表達於葉部細胞，唯其表達爲暫時性的現象，在二個月之後消失（第七章）。另外一方面，胚性癒傷組織也曾應用於粒子鎗基因轉殖試驗，結果成功的得到轉殖植株，且在二種參試的四倍體百合水仙因子型均獲得多個轉殖植株(第六章)。

顆粒型及粉末型這二種癒傷組織均使用於粒子鎗轉殖試驗，而且其後均經過體胚分化的過程而得到體胚，進而成功地獲得完整的轉殖植株。本試驗使用二種質體，各帶有不同的選拔基因與報導基因，第一個質體帶有一個螢火蟲螢光酵素基因，本基因由玉米ubiquitin蛋白質的啓動子(*Ubi1*)所控制，經由粒子鎗把它打進入顆粒型和粉末型癒傷組織裡。粒子鎗打完後次日起，將癒傷組織噴以螢光素水溶液之後，放在螢光檢測器下，螢光酵素基因的表現即可經由檢測器檢查得到。十日之後開始進行選拔，有螢光反應的癒傷組織即予以選拔，其餘則淘汰，藉著螢光檢測器的協助所進行的選拔效果良好。這種選拔法不具有殺傷力，不會對受測的植物細胞造成傷害，檢測完後植物材料可以繼續生長，因此可以作定期的追蹤檢查，螢光表現從癒傷組織階段開始，經體胚，而至完整植株，都可清楚的觀察得到。本試驗結果顯示，利用粒子鎗法轉殖螢光選拔基因，顆粒型癒傷組織的轉殖效率，較粉末型癒傷組織爲高(第六章)。

第二種質體帶有一個抗phosphinotricin(PPT)殺草劑的基因(PAT)，作爲選拔指標，另帶有一個報導基因(GUS)，這二個基因也都是由玉米Ubi1啓動子所控制，經由粒子鎗將其打入顆粒型癒傷組織內。抗PPT殺草劑的選拔法，是將經過粒子鎗處理過後的癒傷組織，移到含有PPT (5 mg/L)的培養基上培養，經過一段期間後，選拔存活的癒傷組織，這些癒傷組織初時呈粉末狀，之後經過體胚分化階段，再長成植株。GUS基因的表達是應用組織化學染色法檢定，轉殖成功者呈藍色反應。本試驗所得到的轉殖百合水仙植株全株器官均呈現深藍色。抗PPT基因撰拔法經証實比螢光基因選拔法的效率高，且操作過程較爲省工(第六章)。

本論文所得到之成果對於百合水仙的栽培，以及基因轉殖研究方面均有助益，葉培植體之作爲微體繁殖單位，對百合水仙而言是一項創舉，由此而可開創出另一種繁殖體系，而加速其繁殖效率。另一種開發成功的胚體化癒傷組織再生體系，不僅可以應用於微體繁殖，還可以應用於基因轉殖。本研究所建立成功的粒子鎗轉殖模式，是一項重要突破，將會促進百合水仙分子育種科技的進展。

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林學詩

CURRICULUM VITAE

Hsueh-Shih Lin was born on 18 March, 1960 in Taoyuang, Taiwan, Republic of China. He finished the primary, junior and senior high school education in the small town Taoyuang from 1966 to 1978. In 1979, he started the academic study in the Department of Horticulture, Chinese Culture University (Taipei city), and received a bachelor degree in 1983. At the same year, he passed a national qualificaton and received a certificate of 'Horticultural Engineer'. From 1983 to 1985 he studied in the Department of Horticulture, National Taiwan University (Taipei) and obtained an MSc degree in Agriculture. After a two-year compulsory military service, he joined the Hualien District Agricultural Improvement Station (Taiwan Provicial Government) as a horticulturist from June, 1987 to September, 1994. During this period he was responsible for plant breeding research on vegetables (Cucurbitaceae), and for the development of *in vitro* culture techniques in order to accelerate propagation and breeding efficiencies in *Ficus, Hemerocallis* and *Lilium*. He was qualified as a governmental officer by a national examination in 1988, and was appointed as a project leader in horticultural research by his institute since 1991. In 1991, he got a fellowship from the Council of Agriculture (Taiwan) and then participated in a three-and-half-month International Course on Applied Plant Breeding in Wageningen, the Netherlands. In 1993, he was recommanded by his institute and awarded a three-year scholarship from the National Science Council (Taiwan), and then he returned to Wageningen and started the research on *Alstroemeria* at the Department of Plant Breeding (WAU) in September, 1994. The results of this research are described in this thesis. At present, he is a 'leave of absence' researcher of the Hualien District Agricultural Improvement Station, and he will return to his institute in September, 1998.