DIAS
report

March 1998

No. 3 • Horticulture

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Ph.D. Dissertation, KVL Copenhagen 1997

Ministeriet for Fødevarer, Landbrug og Fiskeri
Danmarks JordbrugsForskning

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DIAS report Horticulture no. 3 • March1998 • 1st volume

Publisher: Danish Institute of Agricultural Sciences
Research Centre Foulum
P.O. Box 50
DK-8830 Tjele

Tel. +45 89 99 19 00
Fax +45 89 99 19 19

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up to 50 pages 50,- DKK.
up to 100 pages 75,- DKK.
more than100 pages 100,- DKK.

Subscription: Depending on the number of reports sent but equivalent to 75% of the price of sale by copies.

Preface

Imagine our ancestors when they lived in a hunter/gather society. They were forced to pay attention to their environment in order to remember from year to year where they could find high yielding trees or other perennials. So selection of outstanding plants began before agriculture.
When the hunter/gather societies changed to agricultural societies about 14 000 years ago, then, I am sure, farmers wondered why some plants diverged from their neighbours.
The difference between modern varieties of food crops and their wild ancestors show, that farmers, more or less voluntarily, have selected for plants with attractive characters since agriculture began. However, some of these attractive characters change with time, and the selection has to start again in order to fulfil the new demand or need. Breeding can accelerate the formation of new varieties, but many perennials need a certain age before the first flowering, or need to be followed for many years before evaluation and selection is meaningful.
Selection of plants from old plantations, and their subsequent multiplication by *in vitro* cloning is a fast possibility for response to the demand for new varieties.

The main subject of this thesis is cloning of asparagus *(Asparagus officinalis)* and *Alstroemeria x hybrida* by somatic embryogenesis, however, other possibilities for *in vitro* cloning are cowered also.
The elite plants of asparagus were selected from a Danish plantation, however, no research group in Denmark had experience in tissue culture of asparagus. In order to circumvent this drawback we established a collaboration between Dr. Marc Jullien from INRA, Versailles and the Research Group Plant Breeding and Propagation, Årslev - - and I went to Versailles.

The fruit of this collaboration is presented on the following pages, but first I wish to acknowledge the help, patience and flexibility from all members of :

- Research Group Plant Breeding and Propagation
  Danish Institute of Agricultural Sciences, Årslev
Especially I wish to thank: Kell Kristiansen and Kirsten Brandt

- Research Group for Vegetables
  Danish Institute of Agricultural Sciences, Årslev
Especially I wish to thank: Lis Sørensen

- Plant Breeding and Biotechnology
  Den Kgl. Veterinær og Landbohøjskole (KVL)
Especially I wish to thank: Sven Bode Andersen and Brian Dennis

- Laboratoire de Biologie Cellulaire
Institut National de la Recherche Agronomique (INRA), Versailles

- Laboratoire de Biologie des Semences
INRA, Versailles
Especially I wish to thank Marc Jullien and the other members of Laboratoire de Biologie des Semences for their patience in the beginning (when I spoke depressingly little French) and for their patience and encouragement during the writing period.

Holger Ørnstrup
August 1997

Contents

Abstract

The possibilities for *in vitro* cloning of asparagus (*Asparagus officinalis* L.)and *Alstroemeria x hybrida* L. are presented with special emphasis on somatic embryogenesis.
Asparagus: *In vitro* cultivated axillary meristems developed clusters of roots after a short NAA treatment, and from the surface of these roots originated somatic embryos. Isolated embryos developed lines of somatic embryos, which were cultivated without growth regulators for more than two years.
Abscisic acid treatment or desiccation did not improve conversion frequency. Norflurazon stopped chlorophyll synthesis and reduced growth of the somatic embryos, however, conversion frequency was not effected. Improved air exchange and culture on filter paper could improve the fresh weight of mature somatic embryos as compared to embryos cultured directly on the medium without air exchange. Plants have been transplanted to the field where their performances will be followed the next years.
Alstroemeria: Somatic embryos induced from immature zygotic embryos were cultivated in liquid medium without growth regulators. One month culture in liquid medium resulted in a 12-fold increase in fresh weight. Regenerated plants flowered normally. Apical meristems and nodes from *in vitro* grown aerial shoots could produce normal plantlets. Wounded meristems produced between 1.3 - 1.8 plantlets from 40 - 100% of the explants after 58 days.
Somatic embryos were induced from *in vitro* cultivated rhizomes of *Alstroemeria* on a basal medium with the growth regulator combinations: 10 µM 2,4-D + 50 µM BA; 20 µM 2,4-D + 20 or 50 µM BA. Each growth regulator combination produced only few embryos, which germinated without secondary embryogenesis.

Resumé

Med hovedvægt på somatisk embryogenese, bliver mulighederne for *in vitro* kloning af asparges (*Asparagus officinalis* L.) og *Alstroemeria x hybrida* L. præsenteret.
Asparges: *In vitro* dyrkede meristemer udviklede klumper af rødder efter en kort NAA behandling, og fra overfladen af disse rødder udvikledes der somatiske embryoer. Isolerede embryoer dannede linier af somatiske embryoer, der blev vedligeholdt uden vækst regulatorer i over 2 år.
Abscisin syre behandling eller udtørring øgede ikke konverterings frekvensen. Norflurazon stoppede klorofyl syntesen og reducerede væksten af de somatiske embryoer, men konversions frekvensen blev ikke påvirket. Øget ventilation og dyrkning på filtrerpapir kunne øge friskvægten af modne somatiske embryoer, sammenlignet med embryoer dyrket uden luftskifte, direkte på mediet. Planter er blevet udplantet i marken, hvor deres udvikling og produktion vil blive fulgt de kommende år.
Alstroemeria: Somatiske embryoer, der var induceret fra umodne zygotiske embryoer, blev dyrket i flydende medie uden vækst regulatorer. Friskvægten blev i gennemsnit øget 12 gange i løbet af en 4 ugers kulturperiode. Regenererede planter blomstrede normalt.
Apikale meristemer og nodier fra *in vitro* dyrkede skud kunne danne normale planter. Sårede apikale meristemer dannede 1,3 - 1,8 småplanter med en frekvens på 40 - 100%, i løbet af 58 dage.
Somatiske embryoer blev induceret fra *in vitro* dyrkede rhizomer af *Alstroemeria* på et basis medie med: 10 µM 2,4-D + 50 µM BA; 20 µM 2,4-D + 20 eller 50 µM BA. Hvert medie dannede kun nogle få embryoer der dannede småplanter uden sekundær embryogenese.

Prologue

Somatic embryogenesis is a multi-faceted tool with a multitude of applications in modern breeding. Some of these applications are presented in the introduction (Chapter 1).

Modern plant breeding is often referred to as biotechnology, which is a broad term covering much more than the directly plant related subjects. A review of the various biotechnological methods which have been applied to asparagus is presented in Chapter 2, which also introduces the reader to the botanical and genetical peculiarities that challenge the asparagus breeder.

Yield of individual plants in an asparagus plantation is very variable, and the male plants give higher yield and lives longer than female plants. It is possible to measure the yield and quality of spears from individual plants, select the best male plant and multiply this plant. In this way it is possible to plant huge areas of high yielding asparagus plants with well known characters e.g. adaptated for special environments or with improved pathogen resistance. Preliminary results have shown that the average yield from a Danish field of green asparagus can be increased from the normal ≈ 2500 kg/ha for seed propagated plants to more than 7500 kg/ha if the plantation is made of cloned high yielding plants.

Asparagus can be cloned *in vitro* by either somatic embryogenesis or by node culture.

Protocols for somatic embryogenesis have been established for several cultivars of asparagus. However, existing protocols for multiplication by somatic embryogenesis have to be adaptated each time a new cultivar is taken in culture.

The induction of somatic embryos from Danish cultivars of green asparagus is presented in Chapter 3. The reported process involves a very short growth regulator treatment, which induces root development from the explants. Further the first somatic embryos can be isolated from these roots. Efficient conversion of the somatic embryos to plantlets may involve treatments (improved air exchange, high osmotic level) affecting to the water content of the embryos, and in this way mimic the natural desiccation process in seeds. As shown in Chapter 4 the conversion of the somatic embryos of asparagus to plantlets is not simple.

Regenerated plants from somatic embryos have to be transplanted to the field for yield evaluations. Populations derived from somatic embryos are expected to be superior to seed propagated populations because of eliminated variation. Asparagus populations derived from plants multiplied *in vitro* by node culture may be another way to attain the same goal. The node culture technique, however, is not cost efficient and present problems in the root induction process (Chapter 5).

Yield improvement by selection of elite plants is fast and, in asparagus, highly rewarding. However, introduction of new characters in these selected plants by crossing will inevitably also introduce unwanted

characters. Introduction of specific genes can be done by various transformation techniques including microprojectile bombardment and *Agrobacterium*. Although *Agrobacterium*-mediated transformation of monocotyledonous plants is fairly new one of these plants is asparagus. *Agrobacterium*-mediated transformation of asparagus is presented in Chapter 6.

Alstroemeria multiplication by *in vitro* culture was also included in the project.
Normally *Alstroemeria* is cultivated for cut flower production, but it has also a great potential for use as pot plant. However, seed produced plants vary to much, and few varieties is sufficiently low for pot plant production.
Eventually interesting plants from breeding efforts could fairly fast be vegetative multiplicated by somatic embryogenesis. Growth of the somatic embryos in liquid medium represents a further improvement in the multiplication potential (Chapter 7). However, induction of somatic embryos in *Alstroemeria* have only been reported from zygotic embryos. The first indications of the possibility to induce somatic embryos from other tissues as well are presented in Chapter 8.
Asparagus and *Alstroemeria* belong both to the *Liliaceae*, and many similarities can be found, and in our context the most interesting similarity is their common growth by rhizome. The most striking difference is the apparent non-existence of axially meristems on the vegetative shoots of *Alstroemeria* compared to the huge number of axially meristems on *Asparagus* shoots. Cuttings from aerial shoots of Alstroemeria are not able to form new plants under natural conditions. Only by *in vitro* culture is it possible to regenerate new plants from aerial shoots of *Alstroemeria* (Chapter 8).

It is in both asparagus and *Alstroemeria* the enormous potential for clonal multiplication of selected plants that have nourished our interest in somatic embryogenesis.

Somatic embryogenesis --- an introduction

The somatic embryogenesis is a very efficient *in vitro* regeneration process for cloning of plants. It has been attained in hundreds of species by a multitude of techniques (Raemakers *et al.* 1995). The specific technique for each variety is highly empirical, however, some common characters can be found. Induction is generally by a high level of hormones, frequently auxin, and further development and conversion is on media without, or with reduced level of growth regulators. Somatic embryos which multiply by secondary embryogenesis is an efficient tool in modern plant breeding; especially as targets for transformation. Somatic embryogenesis resembles zygotic embryogenesis, consequently fundamental research in zygotic embryo development (seeds) may profit of observations from the somatic embryos and conversely.

A brief introduction to some of the applications of somatic embryogenesis will be presented on the next pages.

Definition

Somatic embryogenesis is the development of embryos from cells of somatic tissue, i.e., those which are not a direct product of gametic fusion. They include e.g. roots, cotyledons, petioles, leaves, and even nucellus.
From the earliest stage the somatic embryos are without vascular connection to the "mother tissue". During development the somatic embryos undergo similar ontogenetic changes as their zygotic counterparts, and finally contain a similar range of reserves at maturity (Bewley & Black 1994).

A few terms in somatic embryogenesis

Some tissues can form somatic embryos directly without an intervening callus phase (direct embryogenesis) when exposed to an appropriate medium. Indirect embryogenesis requires a treatment to initiate mitosis which leads to an undifferentiated cell mass, the callus. The callus is then subjected to further manipulation which leads to the development of the somatic embryos. The first embryos, attained by either direct or indirect embryogenesis, are usually referred to as primary somatic embryos. These primary embryos can produce new "secondary" embryos by a process called secondary (repetitive, recurrent) embryogenesis and in this way maintain a culture of somatic embryos. Cultures producing somatic embryos are often called embryogenic. The expression "embryogenic callus" do cause some confusion as it has been used for any sort of unorganised tissue from which somatic embryos arise. This unorganised tissue can be either a) genuine unorganised tissue (callus) from which primary embryos arise or b) masses of indistinguishable early stage somatic embryos (macroscopically looking unorganised) from which late stage somatic embryos arise. These latter cultures are actually masses of somatic

embryos which proliferate by secondary embryogenesis creating cultures of principally early stage somatic embryos, and a more descriptive name may be "cultures of early stage embryos" (Krikorian 1995). "Proembryos" has been used for very early stage somatic embryos (small globular), they can stick together and form proembryogenic masses (PEMs) (Merkle *et al.* 1990), however, these terms are not clear descriptions and has been avoided in the following text (if not quoted directly).
The term "conversion" cover the developmental process from mature embryo to a small autotrophic plant with normal phenotype, but is often confused with "germination" which describes elongation of the embryo radicle (Redenbaugh 1993 b).
Plants are regenerated when the embryos convert to plants.

The applications of somatic embryogenesis

There are several fields where cloned plants can be valuable.

- Cloning of elite plants. In asparagus the male plants are preferred for female plants because of their higher yield and better survival. Cloning of male plants is a simple way of improving yield in asparagus. Multiplication of elite plants from species which can not be seed propagated e.g. banana and yam is another utilisation of cloning. Many plants can be seed propagated but are very heterozygous so the development of true-to-type hybrids are very difficult or time consuming e.g. asparagus. New varieties from these species can be developed by selecting elite plants for cloning.

- Selected trees and other plants with long life cycles can be propagated more easily by cloning than by seeds.

- Plants obtained by protoplast hybridization or interspecific crosses are by nature rare and need to be multiplied for efficient utilisation in the breeding. Also these plants are genetically heterozygous and often sterile, so without a possibility for cloning the new genetic combinations may be lost.

- Multiplication of new varieties by *in vitro* cloning can reduce the time preceding commercialisation.

- Somatic embryos has been used as explants in numerous transformation experiments and can show transformation frequencies surpassing other explants (Christou 1992, 1996; Firoozabady *et al.* 1994; Raemakers *et al.* 1995).

- *In vitro* selection makes it possible to isolate cells or embryos with desired traits e.g. pathogen resistance (Hammerschmag *et al.* 1995).

The secondary somatic embryogenesis is one of the reasons for the great interest in somatic embryogenesis for propagation. Each new embryo is able to produce several new somatic embryos by secondary somatic embryogenesis. No other propagation method can show similar multiplication potential e.g. in asparagus one somatic embryo can produce 35 new embryos each 4 weeks which gives $3.4*10^{18}$ after one year (Ørnstrup & Jullien 1997), a

single alfalfa embryo can produce $5.31*10^{17}$ after one year (Parrott & Bailey 1993), and in *Manihot esculenta* where a cycle lasts 45 days can be formed $6.6*10^{11}$ embryos in one year (Raemakers *et al.* 1995). However, actually obtained propagation rates are much lower, because of suboptimal handling methods. When the somatic embryos reach a certain maturity they can convert to plantlets by producing root and shoot. The various steps in the culture of somatic embryos will be treated in the next pages.

Induction of somatic embryos

Induction of the first somatic embryos is tissue and species dependent and has been achieved by several treatments. Generally the explant is subjected to a sort of stress. The nature and duration of this stress can vary substantially according to plant variety and explant. Most commonly is used the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) but other strong auxins has been used as well. Often the auxin is accompanied by a cytokinin and it is then the relative proportions between the growth regulators that determines the induction of the somatic embryos. In some cases, especially gymnosperms, cytokinin alone has been able to induce somatic embryos (Nørgaard & Krogstrup 1991; Huetteman & Preece 1993). However, the induction treatment can also consist of e.g. high level of KNO_3 (Ghosh & Sen 1991), high level of sucrose (Kurata *et al.* 1992; Lou & Kako 1995; Taylor *et al.* 1996); sucrose supplied with sorbitol (both at normal 3% level) (Okazaki & Koizumi 1995), or proline supply (Jéhan *et al.* 1994).

Asparagus officinalis is a good example of the variation in the treatments competent for induction and maintaining of somatic embryogenesis in one species. The treatments includes: auxin alone for 10 (Levi & Sink 1991) or 4 weeks (Delbreil *et al.* 1994), auxin and cytokinin in mixture (Levi & Sink 1992; Li and Wolyn 1995) and an inhibitor of GA synthesis (ancymidol) (Kohmura *et al.* 1996). In most of the reports was used the auxin NAA but also 2,4-D has been used (Li & Wolyn 1995). In *A. cooperi* somatic embryos could be induced by increasing the level of KNO_3 from 1900 mgL^{-1} to 2900 mgL^{-1} (Ghosh & Sen 1991).

Although rarely mentioned as the sole determining component the mineral composition of the culture media do have a great influence on the development and maintenance of somatic embryos (Bablak *et al.* 1995; Taylor *et al.* 1996).
Physical factors can influence the induction of somatic embryos as well e.g., induction and maintenance is often performed in the dark (Nørgaard & Krogstrup 1991; Bablak *et al.* 1995; Zhang *et al.* 1995), but regeneration is normally in the light.
Embryo producing callus is generally induced from meristematic or young tissue such as e.g. germinating seeds of rice (Sivamani *et al.* 1996), isolated zygotic embryos of leek (Buiteveld *et al.* 1993) and maize (Ishida *et al.* 1996), immature zygotic embryos from wheat (Weeks *et al.* 1993), scutella of wheat (Becker *et al.* 1994), cotyledons of cucumber (Lou & Kako 1995), meristems of *Hemerocallis* (Krikorian & Kann 1981) and *Iris* (Jéhan *et al.* 1994), cormels of gladiolus (Remotti & Löffler 1995), young flowers of *Iris*

(Jéhan *et al.* 1994) or young leaves of leek (Buiteveld *et al* 1993), sugarcane (Taylor *et al.* 1992), and *Iris* (Jéhan *et al.* 1994) but practically any part of a plant can be used as an explant for induction of callus (review: Ammirato *et al.* 1989; Raemakers *et al.* 1995).

Somatic embryos has been isolated directly from zygotic embryos of wheat (Narender *et al.* 1994), cotyledons and apical tips of carrot (Kurata *et al.* 1992), young leaves of *Quercus suber* (Fernández-Guijarro *et al.* 1995), and several others (Raemakers *et al.* 1995). So the responding tissue may vary from species to species.

Actually the response within a species may vary from one genotype to another e.g. in asparagus (Delbreil *et al.* 1994; Delbreil & Jullien 1994), maize (Duncan *et al.* 1985), and alfalfa (Redenbaugh & Walker 1990, and their references). In maize 91% of 218 inbreed lines were able to produce somatic embryos with varying induction procedures (Duncan *et al.* 1985).

As illustrated by these examples the specific requirements for induction of somatic embryos vary substantially from species to species and must be determined empirically for each plant.

If a callus proceeds the first somatic embryos it must in many cases be subjected to selection, and maintained until the first embryos develop. Generally the first callus which develops from an explant is fairly hard and green, but several callus types can develop from one explant. Taylor *et al.* (1996) describes 4 callus types from *Manihot esculenta* and Bablak *et al.* (1995) describes 5 callus types from *Brachypodium distachyon*. Growth regulator composition and duration of the treatment strongly influences the callus development and patience is important.

Only empirical experience can show which callus type will develop the first somatic embryos, however, generally the goal is to find "friable" callus. This friable callus is often white/yellow and consists of numerous small unattached spherical units (early stage somatic embryos). To avoid confusion the expression "callus" should always be followed by a detailed description. Maintaining the different sorts of calli as distinct lines, following their evolution and identifying the first somatic embryos is a crucial obstacle in somatic embryogenesis. Finally, when the first embryos arise they must be selected and transferred to new media. Regular subculturing is then necessary in order to maintain good growth.

Maintaining of embryogenic cultures

After induction of the first somatic embryos their multiplication by secondary embryogenesis is commonly maintained by lowering the auxin content in the culture media (Raemakers *et al.* 1995). Although the induction of somatic embryos is not understood, it is generally believed that in the presence of auxin the very young embryos within the cell culture synthezise everything necessary to complete the globular stage of embryogenesis but also contain many other gene products which inhibit further progress in embryo development. So when auxin is removed many genes is inactivated and the embryogenesis

program can proceed further (Zimmerman 1993).

Maintaining of embryogenic cultures of asparagus has been performed with auxins (Levi & Sink, 1992; Kohmura *et al.* 1996) but also without growth regulators (Jullien 1974; Delbreil *et al.* 1994 a; Ørnstrup & Jullien 1997). Other embryogenic cultures can be maintained without growth regulators as well e.g., *Hemerocallis* (Smith & Krikorian 1991), alfalfa (Parrott & Bailey 1993), and citrus (Kochba & Button 1974; Yao *et al.* 1996).

Numerous other chemical factors than auxin has been reported to influence the maintenance of the embryogenic cultures, the various cytokinins being the most common ones (Raemakers *et al.* 1995) but also e.g., casein hydrolysate and glutamine which increase the proliferation rate of *Abies normanniana* (Nørgaard & Krogstrup 1991).

Long term maintenance of embryogenic cultures is frequently accompanied by a loss of their ability to progress to mature embryos (Reuther 1990; Zimmerman 1993). It has been proposed that maintenance of the embryogenic cultures on media devoid of growth regulators probably select for further embryonic capacity rather than for the ability to convert into plants (Parrott & Bailey 1993). However, if regeneration-competent callus (Buiteveld *et al.* 1993) or late stage somatic embryos are selected at each subculture the ability to reach maturity and convert to plantlets can be maintained for several years (Reuther 1990; Saito *et al.* 1991; Ørnstrup & Jullien 1997). Especially suspension cultures do often loose their regeneration capacity. This may, however, be attributed to the subculture techniques, as many of these involves selection of early stage embryos by e.g. filtration. These early stage embryos are selected because of their high multiplication potential but their selection do actually establish a strong selective pressure for embryos which bud off new early stage embryos without themselves reaching the mature stage. In this way the maturation ability can be successively lost. This can be circumvented by selecting late stage/mature somatic embryos for subculture (Zimmerman 1993). The multiplication rate of the culture will be lower but it will maintain the ability to produce plantlets.

The filtration process has, however, the advantage that it gives the possibility to collect embryos at specific sizes so that the new population is fairly homogen in development. Further development will then be more or less synchronous. Large quantities of synchronous developing somatic embryos facilitate further work in both propagation and fundamental research (Munksgaard *et al.* 1995).

Conversion of somatic embryos to plantlets

Generally the difficulties in somatic embryogenesis can be divided in two classes: a) Induction, isolation and maintaining of the somatic embryos, and b) conversion to plantlets. Conversion of mature somatic embryos to plantlets with root and shoot includes several similarities with the zygotic embryo development. Zygotic embryo development passes through several stages during seed development and maturation in order

to prepare the seed for dormancy, subsequent dissemination and the later germination with development of the mature plant. During maturation, the seed increases in volume and mass due to embryo development and reserve accumulation. This is a very important phase for the zygotic embryo as well as for the somatic embryo, because it is during this phase, that fully-developed embryos are produced.

Embryo desiccation occurs naturally in most seeds, and has a role in the developmental transition between maturation and germination (Thomas 1993). The conversion of somatic embryos has been improved by various treatments affecting the water potential in the embryo, including polyethylene glycol (PEG) (Brown *et al.* 1989; Attree *et al.* 1991), increased carbohydrate level (Brown *et al.* 1989; Levi and Sink 1990 and 1992) and improved air exchange (Saito *et al.* 1991; Rueb *et al.* 1994). The physiological effect of these treatments, mimicking desiccation during natural seed development, could induce abscisic acid (ABA) synthesis which may then activate ABA responsive genes essential for embryo maturation (see below).

A number of other factors, both chemical and physical, have been shown to have an effect on somatic embryo maturation and conversion. These include, among others, amino acids (Higashi *et al.* 1996), temperature (Li & Wolyn 1996), sucrose (Okazaki & Koizumi 1995), nicotinic acid (Parrott & Bailey 1993), ABA (Brown *et al.* 1989), gibberellic acid (Lapeña & Brisa 1995; Yao *et al.* 1996), pH (Smith & Krikorian 1991), and mineral composition of the media (Jéhan *et al.* 1994). Some of these substances may activate the desiccation responding genes, either by themselves or by secondary products, from their action.

Abscisic acid regulates, among other events, embryo maturation and seed dormancy (Skriver & Mundy 1990). During zygotic embryo maturation ABA levels reach a maximum, suppressing precocious germination and modulating gene expression to induce desiccation tolerance and promoting accumulation of storage products. The ABA concentration generally declines rapidly when the seed dries (Bewley & Black 1994). Late embryogenesis abundant (lea) genes, whose developmental expression may coincide with the rise in endogenous seed ABA level, have been described from various species (Skriver & Mundy 1990; Thomas 1993).

Low osmotic potentials can induce many of the physiological and biochemical effects induced by ABA treatment, and sometimes also induce endogene ABA synthesis (Kong & Yeung 1995). It is even possible that osmotic stress increases the sensivity to ABA. Further, it is likely that the osmotic factor and ABA interact during the seed development (Bewley & Black 1994). Attree *et al.* (1992) found for *Picea glauca* that the optimal culture conditions for maturation, desiccation survival, and plantlet regeneration were 16-24 µM ABA and 7.5% PEG for eight weeks, followed by desiccation to 81% relative humidity. About 80% of the desiccated somatic embryos regenerated to plantlets.

ABA treatment increased the number of mature somatic embryos regenerated from wheat calli, however, their shoot development were suppressed (Brown *et al.* 1989). It is often observed that somatic embryos cultivated on media

containing ABA stop their development at maturity, and then convert when they are transferred to medium without ABA (Skriver & Mundy 1990). The transfer to ABA-deprived medium do then mimic the reduced ABA level in mature seeds.

In general the conversion of somatic embryos is difficult to handle. The somatic embryos do often need a special treatment in order to stop the production of secondary embryos. The elimination of sugars from the medium can stop secondary embryogenesis (Parrott & Bailey 1993). These authors also showed that the carbohydrate and its concentration has a great influence on secondary somatic embryogenesis. Gibberellic acid has improved conversion of somatic embryos from *Fragaria x ananassa* (Wang *et al.* 1984) and *Digitalis obscura* (Lapeña & Brisa 1995). The nature of the conversion treatment can be highly variable but is often related to the water status of the embryo which do not need to be totally desiccated in order to initiate the conversion process. The dehydration process can be slowed by encapsulation of the single somatic embryos in alginate gels as reported by Timbert *et al.*, (1996 a) who found a germination rate of 73% of carrot somatic embryos after a slow (11.5 days) dehydration from 95% to 15% RH.

Artificial seeds

A seed consists essentially of the embryo, the reserves, and a protecting envelope. The reserves, however, may be either mainly in the embryo or in the endosperm according to the species. An artificial seed could be produced if it was possible to give a somatic embryo a protecting envelope and reserves resembling the ones found in normal seeds. Somatic embryos has been encapsulated by several techniques, the most commonly used being alginate encapsulation.
The synthetic seed coat must be non-damaging to the embryo, protect the embryo from mechanical damage during handling, and allow radicle elongation and conversion to occur without delay.
Encapsulated somatic embryos has shown enhanced desiccation tolerance and even improved conversion frequency compared to naked somatic embryos (Timbert *et al.* 1996 a, b). Desiccated somatic embryos, encapsulated or not, can facilitate handling, storage and transport compared to normal moist somatic embryos. Survival of the encapsulated embryos may be improved by supplying the encapsulating matrix with additives e.g. fungicides, mineral nutrients, carbohydrates or symbiotic fungi and bacteria specially adjusted to the specific plants and environments. For further details see Redenbaugh (1993 a).

Somaclonal variation

Somaclonal variation involves all forms of variation encountered in tissue culture. Variation can be either heritable mutations or epigenetic changes. The variation can be considered positive or negative according to the goal chosen by the researcher. Plant breeders dreaming of new variants and propagators clinging to the old ones. Generally plants

regenerated from somatic embryos are identical to the donor plant but a few heritable variations have been found. Ruíz *et al.* (1992) observed 93 plants regenerated from 3 cultivars of *Hordeum vulgare*. Each plant vas regenerated from a different piece of callus. One plant showed visible heritable variation. Nonvisible variation e.g. in the biochemistry is more difficult to evaluate. However, the irone (essential oils) composition of 18 *Iris* clones regenerated from somatic embryos were found identical to their mother-plants (Jéhan *et al.* 1994).

Rueb *et al.* 1994 regenerated rice plants from mature zygotic embryos via somatic embryogenesis and found that spikelet development was disturbed for 2% of the regenerated plants. Two % of the plants with normal spikelets were sterile. Fertility was normal for 56% of the plants and reduced for 42%. Small and thin seeds were produced by 3% of the plants and seeds with twice the size of normal seeds were produced by other 3% of the plants. However, no further genetic study was performed.

Genetic stability of wheat embryogenic tissue cultures has been improved by imitating the nutrient and hormone levels in the zygotic ovule of wheat (Carman 1995). The hypothesis behind this work was that abnormal development may be caused by a variety of physiological stresses, including a) osmotic stresses encountered during culture b) insufficient chemical energy for synthesising macromolecules and for maintaining turgor and correct ionic gradients c) nutrient supplies less complex than those experienced *in situ* confer diversion of energy supplies from growth and development to precursor biosynthesis, and d) slow or variable rates of nutrient absorption across plasmalemmas (Carman 1995). A support of this hypothesis is that, somaclonal variation in alfalfa can be reduced by the use of citrate in the maintenance medium (Redenbaugh & Walker 1990).

Observation for somaclonal variation need long-term experiments, and because of this it is rarely performed above the true-to-type level, but this is a pity because much criticism of somatic embryogenesis is related to the fear of unwanted variation.

However, even natural plant populations can show great variation in chromosome number. Not even has root tips of *Agrostis stolonifera* shown highly variable chromosome numbers in various genotypes, but also within the same plant (Kik *et al.* 1993).

Protoplast culture

Recovering of plants from protoplasts can be highly efficient if the protoplast source is an embryogenic suspension culture (Shillito *et al.* 1989; Attree & Fowke 1993; Panis *et al.* 1993; Yin *et al.* 1993; review: Roest & Gilissen 1993). The logic of this approach is that isolation of protoplasts from tissue that is able to regenerate plants will likely yield protoplast cultures able to form whole plants as well (Shillito *et al.* 1989). Regeneration from protoplast fusions can be facilitated if one of the partners originate from an embryogenic culture (Kobayahsi *et al.* 1988).

Transformation

In order to establish a successful programme for practical plant genetic engineering it is important to develop systems for the recovery of large numbers of transformed plants. The recovery of transformed plants is highly influenced by the transformation technique and the plant tissue used as explant. Tissue from seedlings and zygotic embryos are examples of young (= fast growing) tissue which has proved good starting material for transformation experiments (Klein *et al.* 1988; Becker *et al.* 1994; Hiei *et al.* 1994; Chaudhury *et al.* 1995, Ishida *et al.* 1996), but somatic embryos, which can be produced in large quantities and at defined developmental stages, seems an even better choice. Epidermal or subepidermal origin of secondary somatic embryos (Delbreil *et al* 1994; Taylor *et al.* 1996) is perfect for transformation because it is easy accessible for the transformation agent e.g. metal particles or bacteria. The single cell origin of the secondary embryos eliminates the risk for chimeras because sustained selective pressure on partly transformed embryos restricts the secondary somatic embryos to arise from transformed tissue. In this way, over time, numerous transgenic plants can be produced from a single embryo (Firoozabady *et al.* 1994). Because of this the somatic embryos represent some of the best explants for transformation experiments. However, the high division rate of each cell in the somatic embryo is maybe the most important character for positive results. This has inspired researchers to work with single cells (Arencibia *et al.* 1995) or protoplasts (Toriyama & Hinata 1985; Mukhopadhyay & Desjardins 1994) isolated from cultures of callus and in this way avoid chimaeric transformants. Suspension cultures are especially appreciated as sources of transformable tissue because of their high multiplication potential and large surface area (Finer *et al.* 1992; Vain *et al.* 1993; Firoozabady *et al.* 1994). In some cases the employment of tissue from suspension cultures has even been reported crucial for regeneration of transgenic plants (Schöpke *et al.* 1996). Monocotyledonous plants, however, often needs long time for the establishment of suspension cultures so their use has been restricted to relatively few cultivars of monocotyledons (Finer *et al.* 1992; Guo *et al.* 1995; Ishida *et al.* 1996). However, calli developed from zygotic embryos has been used directly as target for microprojectile bombardment with good results in wheat (Weeks *et al.* 1993) and in rice (Sivamani *et al.* 1996). Transformation can even be performed before the callus stage. Nehra *et al.* (1994) cultured scutella from zygotic embryos of wheat for two days before subjecting to microprojectile bombardment. So the first somatic embryos arose after the transformation event. This is, however, only possible if highly efficient protocols for induction and isolation of somatic embryos has been developed. In the case of wheat the method has proved useful in 10 commercial varieties (Nehra *et al.* 1994). Maize immature zygotic embryos from 6 varieties which were subjected to *Agrobacterium*-mediated transformation yielded phosphinothricin resistant calli from 38% to 90% of the embryos, and

transgenic maize plants from 5% to 30% of the initial embryos (Ishida *et al.* 1996).
Even in the cases where no efficient protocol for somatic embryogenesis has been established the somatic embryos may be the most efficient explant in transformation experiments. This is because the use of the (rare) original somatic embryos can give rise to cultures of somatic embryos which can be used as explants in transformation experiments. Transformed plants can then be regenerated from the somatic embryos. Original explants for transformation may only produce transformed calli without regeneration capacity (Firoozabady *et al.* 1994).

Transformation efficiency is not only related to choice of explant but also highly dependent on the chosen transformation technique. Microprojectile bombardment has been the dominating technique for transformation of monocotyledonous plants for years (review: Christou 1992, 1996). The primary factor that has made particle bombardment the most widely used transformation method is the possibility to target intact explants in the transformation experiments. In this way it is possible to minimise or even eliminate the callus or the suspension culture as target, and thus the accompanying regeneration difficulties. Consequently, breeders can concentrate the transformation efforts on their most advanced plant lines, with significant labour and time savings in the breeding programmes (Christou 1996).
Despite the success and flexibility of the various particle bombardment methods the technique does have drawbacks. Transformation frequency is generally low and the recovery of transgenic plants is fairly labour intensive. Weeks *et al.* (1993) regenerated 2 fertile wheat plants per 1000 bombarded immature embryos, subsequently Nehra *et al.* (1994) improved this frequency to about 20 per 1000 bombarded scutella of zygotic wheat embryos. Further the gene integration process is not understood and sometimes several copies of the foreign gene are inserted in the genome making further breeding work difficult. Inevitably the transformation by microprojectile bombardment needs special equipment and despite substantial simplification the last years (Godon *et al.* 1993) *Agrobacterium*-mediated transformation is technically much simpler. But it has only recently been possible to apply *Agrobacterium* in the transformation process of the monocotyledonous plants e.g. asparagus (Delbreil *et al.* 1993), rice (Hiei *et al.* 1994), and maize (Ishida *et al.* 1996). Contrary to Delbreil *et al.* (1993) who used somatic embryos Hiei *et al.* (1994) and Ishida *et al.* (1996) used zygotic embryos in their transformation experiments. However, plant regeneration was in all three cases by somatic embryogenesis, hence showing the importance of somatic embryogenesis in *Agrobacterium*-mediated transformation of monocots which include some of the most important food crops in the world.

Germplasm preservation

In vitro cultures of vegetatively propagated plants can serve as gene banks, especially at low temperatures.

The low temperatures will reduce growth and in this way reduce handling costs. However, substantial labour is required to maintain an *in vitro* gene bank. Another approach can be cryopreservation. Cryopreserved tissue do not need regularly handling and mutations induced during storage can be neglected. Somatic embryos has in several cases demonstrated their ability to survive cryopreservation e.g. carrot and coffee (Tessereau *et al.* 1994), rice (Cornejo *et al.* 1995), *Musa* spp. (Panis & Swennen 1995), and asparagus (Uragami 1995).

Fundamental research

Embryogenesis is the result of long-term evolutionary processes, and must be highly conserved among higher plants. All plants that reproduce by seed must have all the necessary genes for embryogenesis. Theoretically, for somatic embryogenesis to occur, it is simply necessary to activate the required genes at the appropriate time and in the necessary sequence. Individual cells, with the capacity to undergo somatic embryogenesis, could conceivably be produced by activation of promotor regions of key genes that start the embryogenic process, or alternatively, inactivate repressors which would otherwise suppress the process (Merkle *et al.* 1990). Mutations can give improved ability to produce somatic embryos e.g. in asparagus (Delbreil & Jullien 1994).

The total number of genes in *Arabidopsis thaliana* has been calculated to between 16 000 and 43 500 (Gibson & Somerville 1993). Jürgens *et al.* (1991) estimate that *Arabidopsis* contain approximately 4000 genes essential for embryogenesis. Meinke (1991) evaluate the number of essential genes as low as 500. Body organisation in the zygotic embryo of *Arabidopsis* can maybe be established by only 40 genes (Mayer *et al.* 1991).

Mutations in zygotic embryos of *Arabidopsis* have revealed that whole regions of the embryo body can be deleted by single mutations. For example, *shoot meristemless* do not develop the shoot meristem during embryogenesis and has no leaves; *laterne* do not develop cotyledons but has normal shoot meristems and leaves; in *embryogenic flower* the apical meristem is deleted and a flower meristem develop instead, no leaves is produced only flowers; *gurke* has no apical region; *monopteros* has a normal apical region but the central and basal embryo region is deleted so the embryo do only develop cotyledons and shoot meristem but no hypocotyl or root; *short root* and *hobbit* have abnormal root development but the root meristem is not totally deleted. Mutations perturbing even earlier in embryo development are e.g. *raspberry1* which fails to undergo the globular-heart stage transition, it remains globular and do not develop axis or cotyledons, and *knolle* embryos lack a epidermal cell layer and stay round, without defined apical or basal regions. The *knolle* mutant resembles the carrot somatic embryo mutant *ts11* which develop a defective protoderm and is arrested at the globular stage (De Jong *et al.* 1992; Goldberg *et al.* 1994 and their references; Jürgens 1995 and his references). The reader should consult the literature for extended descriptions, however, attention should be paid to the resemblance of these *Arabidopsis* mutations and the various embryo deformations

encountered in the work with somatic embryos. Mutations homologous to the ones described in *Arabidopsis* can maybe easily be found in abnormal somatic embryos from many other species.

Because the initial stages of zygotic embryo development occur in the ovule, it is difficult to obtain large quantities of the early stages in the embryo development. Somatic embryogenesis has been used as a model system for the study of early embryo development, since large quantities of developmentally synchronised embryos can be achieved (Zimmerman 1993). However, somatic embryos cultured *in vitro* may have developmental patterns different from *in vivo* developed zygotic embryos, especially in the later stages of development, because of their lack of association with ovular tissue and differences associated with *in vitro* culture conditions (Janick 1993). The number of genes isolated from somatic embryos is at the moment fairly low, Zimmerman (1993) mentions 21, but this number may increase rapidly the next years.
Genes for the late embryogenesis abundant (LEA) proteins are among the best studied genes isolated from somatic embryos. They are abundantly expressed late in zygotic embryogenesis and then repressed before dormancy. Lea genes has been described in many plants including *Arabidopsis*, cotton, barley, rice, oilseed rape, and wheat (Dure *et al.* 1989; Skriver & Mundy 1990 and their references; Goldberg *et al.* 1994). The timing of their expression in embryogenesis and their ABA inducibility have led to the suggestion that in zygotic embryos, they play a role in the embryo maturation process and in protection of the embryo during desiccation. In situ localisation experiments have shown that the expression pattern of the lea gene EMB-1 in carrot somatic embryos is similar to its expression pattern in carrot zygotic embryos (Wurtele *et al.* 1993). This should encourage the use of somatic embryos as models for zygotic embryo development.

Changes in protein appearance during embryogenesis has been studied using somatic embryos of e.g. *Digitalis lanata* (Reinbothe *et al.* 1992). The amount of most of the 230 polypeptides chosen for detailed analysis changed during the differentiation of the proembryogenic masses into somatic embryos. Also the proteins liberated into the medium during suspension culture by the developing *Digitalis* somatic embryos changed in a stage specific manner indicating that these proteins could be regarded as marker proteins for specific developmental stages. In carrot, extracellular glycoproteins have been shown to be involved in the acquisition of the embryogenic potential (De Vries *et al.* 1988; De Jong *et al.* 1992; Van Engelen & De Vries 1992).

Transformation by microinjection of single cells in somatic embryos can be used to analyse the developmental route leading to secondary somatic embryos (Lusardi *et al.* 1994). The injected marker can also make it possible to follow the fate of single cells during embryo development so developmental pathways in embryos can be clarified.

As the majority of the worlds agriculture rely on seed propagated crops, it is

clear that seed vitality is essential for future nourishing of the growing world population. The zygotic embryo is the main factor determining seed vitality. Consequently a proficient understanding of the development of the zygotic embryo is essential. The somatic embryogenesis may be one of the main tools in this research.

Conclusions

The propagation by somatic embryogenesis has now reached a level were it is applicable at an industrial level in a few plants as e.g. cacao and coffee.
Application of somatic embryogenesis in the breeding is common for genotype multiplication, regeneration from protoplasts, and plant regeneration after transformation. The importance of somatic embryogenesis may increase in the future as the need for efficient transformation protocols increases. Finally, the somatic embryogenesis may assist the fundamental research in zygotic embryo development (seeds).

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Asparagus Research Newsletter (1997)

Biotechnological methods in asparagus breeding.

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Prologue

In this chapter I have tried to give a short description of the latest asparagus breeding research. Especially I have focused on the possibilities and perspectives in the many aspects of biotechnology which have been studied on asparagus.
The review do not intend to be exhaustive, but I hope it can give an idea of the various tools the modern breeder of asparagus have access to.

Content

Introduction

The main problems in asparagus breeding are related to sex determination and the perennial nature of the plant which gives a long generation cycle. Male plants are preferred, but sex estimation has to await the first blooming. Harvest starts 3 years after sowing but a proper estimation of the potential yield needs recordings from several seasons. Asparagus breeding using traditional techniques is a long process often taking 15 to 20 years from the start of a breeding programme to the release of commercial quantities of seed.
As an open-pollinated species the asparagus plant is very heterogeneous genetically, it do not tolerate strong inbreeding, and heterosis is common. Consequently considerable variation exists in yield of individual plants of the same variety.

Origin

Asparagus officinalis L. is a perennial monocotyledon which belongs to the Liliaceae.
Asparagus is a large genus (about 150 species) of herbaceous perennials and tender woody shrubs and vines, grown most as ornamentals, but one, *Asparagus officinalis* is grown as a vegetable. Before it was used for food, asparagus was used as a medicine for almost anything from the treatment of bee strings to heart trouble, dropsy and toothache. The Greeks who gave asparagus its name, cultivated asparagus as a luxury vegetable as early as 200 B. C. According to Moreau and Zuang (1977) the first reports are from Egypt and Greece (but this was maybe the species *Asparagus acutifolius*). The same authors quote Caton for reporting that the romans cultivated *Asparagus officinalis*. Although *A. officinalis* is the only asparagus species cultivated as a vegetable, even now rural people from Spain to Greece collect and eat the young spears of the species *Asparagus acutifolius*, which grows wild in the coastal regions of the Mediterranean. Here we also find *Asparagus maritimus* which is the wild species most similar in appearance to *A. officinalis*. Most of the cultivated "varieties" of asparagus today are merely strains of *A. officinalis*, with little to separate them morphologically one from another.

Botany

The perennial part of the asparagus plant is the crown, a rhizome like underground structure consisting of buds and storage roots along with absorbing rootlets. Every spring young stems, the spears, emerge from the rhizome. They represent the edible part of the plant and are collected every day for approximately two months. After the harvest period the 2 m high aerial shoots are allowed to grow and develop "branches" covered with needle-like cladophylls (leaf-like structures morphologically related to stems), while the leaves are reduced to small scales along the stems. The foliage is known as the fern.

Asparagus flowers are borne on young shoots and reach anthesis before maturity of the shoots and cladodes. One plant can produce approximately 400 solitary flowers, borne two per node, one at each side of a lateral branch or cladophyll (Lazarte and Palser, 1979). On female

plants the first blooming is seen a little later (30-60 days) than on the male plants sown at the same time. This allows the female plants to support the fairly big energy loss used for seed production. Mature male and female plants will flower simultaneously.

In Denmark field grown plants will flower about 2 years after sowing, in France 14 - 16 months after sowing (Moreau and Zuang, 1977), and under tropical climates flowering can be seen approximately 100 days after sowing. Asparagus has male, female and hermaphroditic plants (a dioecious plant).

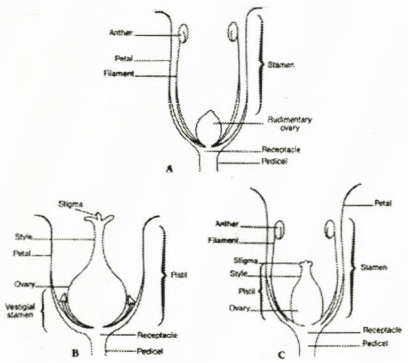

Figure 1. (A) Normal flower of male asparagus. Note rudimentary sterile ovary. Anthers are yellow and produce much pollen. (B) Normal flower of female asparagus. Note rudimentary stamens. Anthers are white and do not produce pollen. (C) Androgenous flower of andromonoecious asparagus. Drawing by J. J. Kinelski from Ellison (1986).

Male plants can produce hermaphroditic flowers with rudimentary pistils; however, sometimes the female organs are sufficiently developed for producing seeds. These berry-bearing male plants are called andromonoecious, and occur at a frequency of 0-1% of male plants. Male andromonoecious plants can be divided into 4 groups: a. plants producing no berries; b. plants producing 1 - 10 berries; c. plants producing 10 - 100 berries; d. plants with > 100 berries per year (Franken, 1970). Functional hermaphroditic flowers are (probably) self-pollinated, and can set one or more viable seeds. Female flowers have rudimentary stamens, none of which have been known to produce pollen (Ellison, 1986). The fruit, which contains 1-9 round black seeds, is a reed berry (5-10 mm diam.) when mature.

Flower induction and sex determination

The fact that the male and female plants differ in several characters of horticultural interest, makes the inheritance of sex expression very important for the breeder. Male plants are more desirable for commercial production than female ones because of greater yield, vigour and longevity. Female and male plants can only be distinguished by the sex of the flowers. So early induction of flowers in seedlings will enable earlier distinction of the sex. Flower induction by temperature and daylength has been investigated by Thevenin, (1967) and Hsung, (1985) but they were not able to uncover the determining combinations for flower induction. However, procedures for flower induction by a chemical treatment has been established. Abe and Kameya, (1986) used the herbicides Atrazine and Diuron; Mizonobe *et al.*, (1991) used the anticytokinins s-triazine and carbamate to induce flowering in 92% of the

seedlings (if germinated at 40 °C), and recently Hara *et al.* (1992) obtained 80% of flowering by anilide and benzamid derivatives.

Naturally male and female plants occur in about equal numbers.

Male plants can be heterozygotic (Mm) (generally andromonoecious) or homozygotic (MM) (occasionally andromonoecious) and have flowers with mostly rudimentary pistils containing degenerated ovules. Female plants are homozygotic (mm) recessive; their flowers have stamens with collapsed anthers lacking pollen (Lazarate and Palser 1979).

Chemical flower induction can be used to select for male plants for further production, but can also be used on a small seed lot to establish the sex ratio in a crossing; with the final goal to deduce whether the male parent were a normal male (Mm) or a super male (MM). If the seed lot contains only male plants (Mm) then the pollen donor must have been a super male (MM). Progeny of the heterozygotic (Mm) male will segregate male (Mm) and female (mm) in a 1:1 ratio.

Sneep (1953) proposed the possibility of using andromonoecism in the breeding of uniform male hybrids by crossing female (mm) inbreeds with supermale (MM) inbreeds. But as females (mm) can not be selfed because of lack of viable pollen Sneep (1953) proposed selfing an andromonoecious plant (Mm), selecting an Mm in S1 for selfing, and continuing the selfing series to homozygoty. The inbred females from these selfings can be used as parents. To produce inbred supermales (MM) he proposed a similar selfing program beginning with an andromonoecious (Mm) plant. A faster method for obtaining inbreed plants is by chromosome doubling of haploids obtained by anther or pollen culture. The megagamatophyte can be cultured if the female plant is especially interesting.

High seed production on andromonoecious plants can drain the male plant for assimilates as on the female plants. So andromonoecism must be avoided in order to maintain the superiority of the male plants. Only males (Mm), or even better, supermales (MM) with very few berries (less than 10) should be used as pollen donors in practical breeding programmes (Franken 1970).

Morphological evidence indicates that sex differentiation in asparagus consists essentially of selective abortion of gynoecium or androecium of initially hermaphroditic floral primordia occurring on genotypically determined male or female individuals. The abortion occurs in pollen-mother cells and anthers on females and in megaspore-mother cells of the ovary in males (Lazarte and Palser 1979; Bracale *et al.* 1991a). Löptien (1979) has shown that the genetic system controlling abortion of male or female organs is apparently monogenic (possibly a bipartite gene) associated with chromosome pair L5 (at least partly). The modification of the developmental pathway is accompanied by changes in relative abundance of auxin and cytokinin (Bracale *et al.* 1991a). Lazarte and Garrison (1980) were able to induce a higher percentage of hermaphroditic flowers (actually suppress the abortion of style and ovary) on male plants by applying the cytokinin PBA; but the fruits were seedless. On female plants the same authors were

able to induce sterile anthers by applying gibberellins (GA3) alone or together with cytokinins (PBA). In the future it will probably become possible to induce hermaphroditic flowers on selected plants to be used in the breeding work.

In vitro culture techniques

Cloning

Cloning of selected plants can have two goals for a breeder a) multiplication of plants used in further breeding (e. g. selected parents for crossings and seed production) or b) multiplication of selected elite plants at a commercial scale.
Asparagus has a low multiplication rate using conventional methods for vegetative propagation. One plant (or crown) can be divided in 2-4 new plants per year, and in addition the injured surface is a site for pest invasion. Thus this technique can produce very limited numbers of plants. Potted plants kept under high relative humidity can develop aerial crowns on the stems (Yang and Clore; 1973 and 1975). When they enlarge these aerial crowns will produce roots and shoots, and can be planted separately. However, division of crowns and production of aerial crowns are not economically feasible. The only suitable method for cloning of selected plants are by the *in vitro* techniques.
Essentially there are two techniques for *in vitro* multiplication of asparagus plants: Shoot culture or somatic embryogenesis.

Shoot culture

In vitro propagation of selected asparagus plants by the shoot apex (meristem) were reported by (Hasegawa *et al.* 1973; Murashige *et al.* 1972; Takatori *et al.* 1968). But the excision of the apex or the meristem is very difficult and time consuming. Another *in vitro* technique using shoots emerging as axillary branching from nodal cuttings has been reported by several authors (Doré 1975 and 1988; Yang and Clore 1973). The shoots emerging from the nodes are cut of several times during the culture, and slowly a rhizome will form at the base of the shoots. When sufficiently large the rhizomes can even be divided *in vitro*, hereby improving the multiplication rate. Subsequently the rhizome will produce roots. The technique can be considered quite efficient and is even used for commercial production of asparagus plants in France and New Zealand. However, the high demand for manpower during the *in vitro* production phase is a limiting factor in the commercialisation of these plants. The recalcitrance of *in vitro* multiplied shoots to form roots *in vitro* (before transplantation) seems now solved (Shigeta *et al.* 1996). These authors used gellan gum (8 g/l) and glucose (4-5%) in the rooting medium to improve the rooting frequency.
Cloning of single plants for breeding, by *in vitro* multiplied shoots, was first reported by (Yang and Clore 1973) and is now used in many breeding programs. A review of micropropagation of asparagus can be found in Desjardins (1992).

Somatic embryogenesis

The rooting problems encountered in the *in vitro* cultures of nodes and rhizomes of asparagus have directed much interest towards somatic embryogenesis.

Somatic embryogenesis is a very efficient cloning technique and early stage somatic embryos can be cultivated in liquid culture, which in adequate conditions can yield suspension cultures with an enormous multiplication potential. Further the somatic embryos are very good starting materials for genetic transformation.

Somatic embryos from *Asparagus officinalis* has been induced from several explant sources: hypocotyls (Wilmar and Hellendoorn 1968), stems (Reuther 1977), cladophylls (Harada 1973) and cladophyll cell cultures (Jullien 1974). In some cases long-term embryogenic cultures can be obtained (Jullien 1974; Delbreil *et al.* 1994a). These cultures can be maintained on hormone-free medium and produce, by secondary embryogenesis, large quantities of somatic embryos in all developmental stages (Delbreil *et al.* 1994a). The fact that they can be maintained without growth regulators reduces the possibilities for variation induced by long-term growth regulators. However, Delbreil *et al.* (1994b) presents evidence for an *in vitro* induced mutation which improves the ability of asparagus plants to produce somatic embryos when taken in culture. No other variation were detected in the regenerated plants.

Reuther (1990) used the ploidy level to examine variation in callus and the regenerated plants. He found that callus with a high regeneration potential was diploid, and callus with reduced regeneration ability had a highly variable ploidy level. Actually all plants regenerated from supermale callus lines were diploid. Female callus lines were found to have a higher ploidy variation, and 10% of the regenerated female plants were polyploid. However, ploidy changes are actually fairly big rearrangements in the genome and much variation can not be detected with this technique. Molecular biology techniques holds much better possibilities for estimation of variation.

Synthetic seeds composed of encapsulated somatic embryos has been developed in order to facilate handling, and improve the possibilities for storage of the somatic embryos. Alginate encapsulated somatic embryos from *Asparagus cooperi* (Baker), which had been stored for 90 days at 2 °C, were able to germinate to plants at a frequency of 8.3% (Ghosh and Sen 1994). This conversion frequency is too low for commercial use but sufficiently interesting for further study.

Protoplast culture

Regeneration of plants from asparagus protoplasts has been reported (see Dan and Stephens (1994) for review). Efforts in protoplast research are generally justified by the possibility for introduction of foreign genes by protoplast fusion. Kunitake *et al.* (1996) were able to hybridize protoplasts of *Asparagus officinalis* and *Asparagus macowanii* by electrofusion, and confirmed the fusion by isozyme and RAPD analysis of the regenerated plants. However, a big

drawback in protoplast fusion is the unpredictable result. Introduction of isolated genes can overcome this obstacle, and as protoplasts by nature are composed of one single cell, they are perfect subjects for transformation. Asparagus protoplasts has been transformed by electroporation (Mykhopadhyay and Desjardins 1994). The largest drawback in the work with protoplasts is their slow regeneration to plants (at least 6 months has to be calculated from protoplast isolation to plantlet). However, May and Sink (1995) were able to reduce the time from protoplast isolation to plantlet to 6 weeks by using protoplasts isolated from embryogenic cell suspensions.

Somaclonal variation

Micropropagation can improve the survival of genetically altered cells arising spontanously in the somatic tissue, especially if the micropropagation methods involves a callus phase (Reuther 1990). For instance it allowed the selection of a highly embryogenic mutant useful in fundamental studies of hormone action (Delbreil *et al.* 1994a). This somaclonal variation can be regarded as a positive or a negative effect of micropropagation. If the purpose is to clone a selected plant, somaclonal variation will be a highly unwanted supplementary effect. However, in mutation breeding the somaclonal variation can be a valuable tool for gaining variation (Reuther 1990).

Gene banks

World-wide the genus *Asparagus* includes about 300 species.

Only *Asparagus officinalis* is cultivated as a vegetable, but other species are cultivated for their ornamental value as well. As the technical possibilities for interspecies crosses increases the future breeders will certainly appreciate collections of closely related species (genus collections).

Many characters (e.g. pathogen resistance and salt- and cold tolerance) could be improved by inter species crossings.

A. densiflorus cv. Sprengeri and cv. Myersii received the lowest ratings for susceptibility to *Fusarium oxysporum* f. sp. *asparagi* and *F. moliniforme* among four tested species of *Asparagus* (Stephens *et al.* 1989). *A. asparagoides, A. compactus, A. densiflorus* cv. Myers, *A. densiflorus* cv. Sprengeri, *A. larcinus, A. verticullatus* and *A. virugatus* have been classified as being highly resistant to *Stemphyllium* sp. (Bansal *et al.* 1986). *A. densiflorus* cv. Sprengeri has been classified as being immune to *Puccinia asparagi* (Thompson and Hepler 1956). *A. densiflorus* cv. Sprengeri was found to be resistant to *Phoma asparagi* (Tu *et al.* 1988). So there are several (known) possibilities for advantages from gene banks. Future pathogen pressure and consumer demands will surely change, and the gene banks will be a valuable toll for satisfying new demands. *In vitro* culture and molecular biology techniques could be used to isolate and transfer specific genes to species within the same genus.

Individual plants of *A. officinalis* and other species from temperate climates can be maintained in the field, but special care has to be taken to tropical and subtropical species. In

the asparagus collection of the USDA, ten tropical/suptropical species are currently maintained as clones in the greenhouse (Uragami 1995). A special problem of maintaining asparagus plants in the field is that the individual plants can grow into one another. In order to avoid this a sufficiently large area is required per plant. Self sown seedlings from lost seed have to be completely removed every year.

In vitro storage

In vitro propagation and storage can overcome most of the problems encountered when maintaining germplasm in the field. Germplasm cultured *in vitro* can be maintained free of pathogens (fungi and bacteria), and with a little extra effort can virus be eliminated from the starting material before storage. Pathogen free germplasm will facilate international exchange of germplasm. This can be a very strong argument for *in vitro* culture.

However, new problems will arise from the *in vitro* culture, the greatest problem will be loss of interesting properties as a result of genetic instability during long-term culture. This is normally negligible but can not be excluded.

In vitro storage can be performed as cultures of 1) meristems or axillary buds from shoots that has been grown *in vitro*, 2) crowns cultivated *in vitro*, 3) somatic embryos or 4) cultures of callus.

Cryopreservation

Cryopreservation is a possibility for long-term preservation of interesting germplasm without danger of genetic instability. Cell lines of embryos or callus can be stored before they loose their regeneration ability. Mutated or genetically manipulated germplasm can be stored safely for future use. Asparagus has been subjected to several studies on cryopreservation (see Uragami (1995) for a recent review). The survival of cryopreserved asparagus tissue is generally very high.

The percentage of somatic embryos surviving cryopreservation ranges from 48% (Uragami *et al.* 1989) to 80% (Uragami 1991). The survival rate of embryogenic callus after 6 months storage were 61% (Uragami 1991). Plants were regenerated from 70% of nodes with axillary buds (Uragami 1995) and 95% of multiple bud clusters from a meristem culture produced shoots after a cryopreservation treatment (Khomura *et al.* 1992).

Previously cryopreservation of asparagus tissue involved several steps including various forms of preculture and desiccation treatment, use of cryoprotectants, more or less controlled decrease of temperature and later rise of temperature. However, as the knowledge and experience increases the treatments will be reduced and standardised, and the need for expensive equipment for temperature control will decrease. Uragami (1995) placed the desiccated tissue in a 1 ml plastic tube and transferred the tube directly to liquid nitrogen; thawing were performed in air at room temperature (no special equipment were needed). With the development of less complicated cryopreservation methods their implication in germplasm storage will surely increase.

Pollen

Storage of pollen is especially important for the breeder, as it reduces the need for simultaneous blooming in the parents of specific crosses.
Snope and Ellison (1963) found that lowering temperature and relative humidity (RH) increased pollen life considerably. Pollen viability diminished rapidly after 2 weeks at 20°C with 75% RH, after 8 weeks at 1°C with 75% RH and after 8 weeks at 20°C with 15, 45 and 75% RH. However, viability was undiminished after 60 weeks at -20°C with no control of RH and at 1°C with 15% and 45% RH.
Ellison (1986) stored pollen in tightly closed vials in an ordinary domestic freezer, but although the pollen remained viable after more than 6 months he recommends the pollen to be replaced each 6 months. Falloon, however, stored pollen at -18°C for 18 months without problems.

Seed

Asparagus plants are mostly propagated by seeds. However, since asparagus is an almost completely allogamous plant, unique combinations of genes of individual plants cannot be preserved through their seeds.
Low germination is a common problem with asparagus seed, but most species can be preserved as seeds (Uragami 1995). Seed from red-ripe fruit is superior in germination to seed from less mature bronze-coloured fruit (Scheer *et al.* 1960). When harvested the berries are crushed in order to separate berry skins and pulp from the seed, which is then spread out on screens to dry overnight.
In the asparagus collection of the USDA, asparagus seeds were stored at 4°C and 40% RH until 1991, when they were mowed to a new storage facility maintained at 4°C and 25% RH. Seeds stored since 1983 had a germination rate of 57-69% in 1991 (Uragami 1995).
Pathogens on seeds can be a problem in the international exchange of germplasm. Fusarium spores are common on asparagus seed, and treatments with either fungicides or disinfectants is a must (Ellison 1986). Asparagus virus 2 (AV 2) is transmitted through seed, and up to 67% of the seed can be infected (Paludan 1964).

Genetics and molecular biology

Asparagus officinalis has 10 chromosomes (2n = 20), and haploid genome size is around 1.8 x 10^6 kbp. (Bracale *et al.* 1991a).

Monoclonal antibodies

Monoclonal antibody technology allows the production of specific antibodies starting from an crude, impure, heterogeneous protein (immunogen) preparation and has proved to be the only method useful when antigenic proteins have not been previously isolated (Bracale *et al.* 1991b).
Isolation of antibodies against specific parts of the plant (e. g. the male and female flowers) can be used to screen an expression library for clones expressing the proteins of interest. Monoclonal anther specific antibodies has been raised against anti-

genes from anthers and pollen cells from male asparagus flowers (Bracale *et al.* 1991b).

When producing these monoclonal anther specific antibodies Bracale *et al.* (1991b) first isolated crude protein extracts from unopened anthers, leaves (cladophylls) and young roots of male asparagus plants, then injected the protein extract from the anthers in mice and the antibodies raised here were then subjected to the three protein extracts from anthers, leaves and roots. Proteins from leaves and roots representing housekeeping proteins, present in both vegetative organs and anthers. Only antigens showing positive reactions against anther extracts and no detectable reactivity with leaf or root proteins were selected.

Besides the screening of an expression library in search of the related genes, the availability of probes signalling the expression of specific male antigens can be very important for monitoring the developmental pathway of male inflorescence, particularly when this development can be altered. As mentioned elsewhere in this report the developmental pathway of asparagus flowers can be altered by exogenous treatments with hormones.

Isozymes

Isozyme systems are restricted in their utility by the number of enzyme systems that can be visualised; and by the fact that isozyme expression can be influenced by environmental conditions or management practices. Using 7 enzymes Geoffriau *et al.* (1992) could only find specific bands in 4 of 21 accessions of *A. officinalis.* However, they were able to estimate some of the genetic variation within the 21 accessions. Three groups were estimated as belonging to either a) wild type (collected in Turkey, not cultivated) or b) a highly improved group, including some of the most interesting cultivated varieties or c) an intermediary group including traditionally grown varieties from Spain which have been subjected to very little or no breeding. Not surprisingly the variation within a group were found to be much greater in the wild population from Turkey and the intermediary population from Spain than in the cultivated accessions. Further subgrouping could be done as well (e.g. distinguish the French and American accessions) and deducing the origin of the Taiwanese population to be American. However, the used isozymic markers were concluded not sufficiently to identify individual asparagus cultivars.

DNA

DNA-based markers offer a number of advantages over isozymes and other biochemical methods in their distinctness. Firstly, the DNA sequence of a organism is independent of environmental conditions or management practices. Secondly, all sorts of tissue at any stage of growth has the same DNA content; plants can actually be screened at the seedling stage - before the trait is expressed. Using restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) markers the resolution in the genetic evaluation can be greatly improved. Hu and Quiros (1990)

used RAPD markers to discriminate among 14 different broccoli and 12 different cauliflower cultivars.
Also characters which are a result of a joint action of several genes can be investigated by RFLP or RAPD markers. Such characters are often referred to as polygenic or quantitative. The underlying loci are called quantitative trait loci or QTLs. Examples are: yield, pathogen resistance, drought tolerance, harvest date and quality. These characters are much more difficult to handle in breeding programmes than single gene traits. The search is made for associations between the segregating RFLP/RAPD markers and the character of interest.
In backcross breeding the DNA-profiling techniques can serve as well. Plants which have the desired trait and a high level of recurrent parent genome can be selected for further backcross breeding. In this way the number of generations required to eliminate unwanted genes linked to the desired trait can be substantially reduced. Tanksley *et al.* (1989) show an example of RFLP assisted selection where the number of generations, needed to reduce the size of the (unwanted) linked DNA to 2 cM, are reduced from approximately 100 generations with normal breeding techniques to 2 generations with RFLP assisted selection. The reviews of Morell *et al.* (1995) and Tanksley *et al.* (1989) supplies further details of the possibilities in DNA profiling.

Isolation of specific genes

Until now very few genes has been isolated in asparagus. However, the isolation of the wound induced promoter AoPR1 (Warner *et al.* 1993), has opened the possibility for study of pathogenesis related (PR) reactions in asparagus. AoPR1 can even be the harvest-induced promotor for the gene encoding asparagine synthetase which was isolated by Davies and King (1993). Harvest-induced genes are especially interesting for asparagus because the general quality of the harvested spears declines rapidly after harvest. If it is possible to down regulate key genes induced during, or shortly after harvest, then the quality of the commercialised product can probably be improved.

Genetic engineering

Methods for transformation of dicotyledonous plants by *Agrobacterium* have been well established, but not for monocotyledonous species, except in a few cases (Delbreil *et al.* 1993; Hiei *et al.* 1994; Ishida *et al.* 1996). Although the number of reports using Agrobacterium for transformation of monocotyledonous plants have increased the last 2-3 years, the most widely used (and most efficient) method for transformation of monocotyledonous plants is still the bioballistic process. But compared to the bioballistic process: 1) *Agrobacterium* can introduce much bigger pieces of foreign DNA in the host cell hereby increasing the potential future use, 2) Genes introduced by *A. tumefaciens* will normally occur in single to few copies so the further breeding work will be facilitated compared to the multi copies often found if the bioballistic process is used and 3) the *Agrobacterium* mediated gene transfer is a natural biological process which minimises the alteration of

the steady state in the host cell compared to the relative large physical damages imposed when the projectile hits the potential host cell.

Asparagus officinalis is one of the monocotyledons plants which have been transformed by several procedures, including *A. tumefaciens*. Hernalsteens *et al.* (1984) isolated tumour tissue, from young asparagus stems infected with the oncogenic C58 strain of *A. tumefaciens*. Bytebier *et al.* (1987) extended the study to include a non-oncogenic strain and selected transformed tissue on kanamycin containing medium. The transformation was confirmed by DNA-hybridization (Southern), and kanamycin resistant plants were regenerated. Conner *et al.* (1990) transformed Asparagus with three species of *Agrobacterium* (*A. tumefaciens*, *A. rhizogenes* and *A. rubi*). This work showed that several species of *Agrobacterium* are able to mediate transformation of *Asparagus officinalis*. Meristems from seedlings and whole zygotic embryos are examples of young (= fast growing) tissue which could be good starting material for transformation experiments, but somatic embryos, which can be produced in large quantities and at several defined developmental stages, seems an even better choice. Somatic embryos from *Asparagus officinalis* can as mentioned earlier be induced *in vitro* from several explants sources. Delbreil *et al.* (1993) found that cylindrical somatic embryos (800 - 1600 µm) could be transformed by *A. tumefaciens*. Some of these transformed somatic embryos developed a line of somatic embryos from which plants could be regenerated.

The particle gun has been used as an alternative to *Agrobacterium* mediated transformation of asparagus. Perri *et al.* (1994) confirmed introduction of a plasmid containing both the *gus* and the *npt*II gene, to an embryogenic cell suspension by recovering kanamycin resistant calli and by performing X-Gluc coloration and PCR analysis of the resistant calli. Plants were not regenerated.

Asparagus protoplasts has been transformed by electroporation (Mukhopadhyay and Desjardins 1994), but plant regeneration was not reported. So only *Agrobacterium* mediated transformed asparagus tissue has been able to regenerate plants. But it is just a matter of time before an efficient technique for transformation of asparagus will be reported.

Haploid plants

Gene markers

Haploid seedlings were detected by Basset *et al.* (1971), who used a recessive seedling marker gene (red basal stem) to detect parthenogenesis in female plants. When red-stalk female plants were crossed with a homozygous dominant green-stalk male, any seedlings in the progeny that showed the recessive red-stalk character were very likely to be haploid.

Anther and pollen culture

Anther culture is potentially a powerful tool for producing haploid or doubled haploid asparagus plants and has been reviewed by Doré (1990). Anther culture has been used to produce homozygous supermales,

which has been used as pollen donors in the production of all-male hybrids.

The first reports of anther culture on asparagus plants reported callus production and plant regeneration from this. However, callus can originate from somatic tissue of the anther rather than from pollen microspores (Hondelmann and Wilberg 1973) giving rise to plants of parental genotype (Doré 1979). Even a test for chromosome ploidity can not safely indicate weather a tissue is of somatic (diploid) origin or microspore origin, as all but a few (2.75% to 8.25%) haploid cells involved in callus formation undergo spontaneous chromosome doubling to produce diploid or polyploid lines (Falavigna *et al.* 1985; Tsay and Hsu 1985). When not occurring spontaneously at the callus stage in anther culture, conversion to the diploid stage appears to be a difficult step. However, chromosome duplication has been reported by several authors (Doré 1976 and 1990; Reuther 1990; Skiebe *et al.* 1991). Beyond the tetraploid level, asparagus plants are completely sterile and short lived; six n chromosomes is the highest ploidy level observed for anther derived plants successfully transferred to soil (Doré 1990).

Morphological markers could provide a good method for evaluating anther derived plants and determining heterozygosity or homozygosity. However, very few of such markers exist in asparagus, so many workers have used the sex factor as a marker. But as male plants can be both heterozygotic (Mm) and homozygotic (MM) a test crossing must always be performed. Isozyme markers in this case would be superior to morphological markers (no possibility for subjective evaluation of morphological characters), and without the long wait for blooming.

But the simplest solution is to use pollen as explants for production of homozygous supermales, excluding any possibility of diploid tissue from the "mother" plant. Recently this has become possible (Zhang *et al. 1994*).

Haploids from polyembryogenic seeds

Haploid plants can occur spontanously from twin seeds. These are generally a result of parthenogenesis which happens at very low frequency. In asparagus Thévenin (1968) have estimated the frequency of polyembryology (twin seeds) to 0.22 %. The frequency of haploids was estimated to 2 % of the twin seeds, giving approximately 1 haploid among 25000 seeds. More than 100 haploids obtained by Thévenin (1968) were all female. Marks (1973) germinated seeds from open pollinated single female plants and found that the frequency of haploid plants among twin seeds depended on the plant used as source for the seeds. As a natural source of haploids twin seeds are very interesting, but considering their low frequency haploids from twin seeds are now replaced by anther and pollen culture as sources for haploid plants.

Conclusion

The modern asparagus breeder has access to a large array of tools, which are constantly evolving. The *in vitro* culture techniques (protoplast culture, shoot culture, somatic embryo-

genesis, anther and pollen culture) are now almost customary. The efficiency can be improved in all fields, but the results are now sufficiently good for use in practical breeding. Isolation of specific genes and transformation techniques optimised for asparagus are in their infancy. However, these techniques evolves very fast, and as duration and extent of asparagus breeding programmes can be reduced substantially their implication will be further advanced.

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Somatic embryos obtained from *in vitro* root cultures of asparagus.

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Key words: *Asparagus officinalis*, cloning, habituation, *in vitro* multiplication, somatic embryogenesis.

Abstract

Somatic embryos were obtained from *in vitro* root cultures of *Asparagus officinalis* L. The initial explants consisted 1-2 mm axillary shoots, from young spears, that were subjected to a 9 day pulse of 53.7 µM NAA and then cultivated without growth regulators for 12 - 14 weeks. During this period the explants developed clusters of roots and from the surface of these roots somatic embryos emerged. Isolated single embryos in turn gave rise to secondary/tertiary somatic embryos, which were cultured without growth regulators for more than two years. The potential multiplication capacity of this scheme is very high. Plants have been transplanted to the field where their performance will be followed.

Abbreviations: BA - 6-benzylaminopurine GA - gibberellic acid, NAA - naphthaleneacetic acid.

Introduction

Asparagus officinalis L. was among the first plant species to be cloned by somatic embryogenesis about 30 years ago (Wilmar & Hellendoorn 1968). Since then several protocols for somatic embryogenesis in asparagus have been reported. Somatic embryos have been induced by treatment with auxin alone for 10 (Levi & Sink 1991) or 4 weeks (Delbreil *et al.* 1994), with auxin and cytokinin (Levi & Sink 1992; Li & Wolyn 1995) or with an inhibitor of GA synthesis (Kohmura *et al.* 1996).

Embryogenic cultures of asparagus have been maintained with auxins (Levi & Sink 1992; Kohmura *et al.* 1996) but also without growth regulators (Jullien 1974; Delbreil *et al.* 1994).

Hypocotyls (Wilmar & Hellendoorn 1968), stems (Reuther 1977), cladophylls (Harada 1973) and cladophyll cell cultures (Jullien 1974) all have been used as explants. Long-term embryogenic cultures obtained by Jullien (1974) and Delbreil *et al.*

(1994) were maintained on medium without growth regulators and produced, by secondary embryogenesis, large quantities of somatic embryos in all developmental stages. A detailed description of *in vitro* culture and somatic embryogenesis in asparagus has been given in several reviews (Reuther 1984; Desjardins 1992; Levi & Sink 1995).

Here we report a protocol for the induction of somatic embryos from *in vitro* produced roots of asparagus, and the maintenance of these cultures on medium without growth regulators.

Materials and methods

Plant material

Three high yielding male plants (nos 15, 19, and 24) were selected from a three year old plantation of the seed grown green variety "Aarslev 270" (Sørensen & Thuesen 1992). From a similar plantation of the green variety "Aarslev 136" one plant was selected (no 132) (Sørensen & Thuesen 1992). These selected plants were used for initiation of the *in vitro* cultures.

In vitro shoot cultures

Shoot cultures were started from node explants of secondary branches of the selected field grown plants. These node cultures were maintained on a basal medium (Delbreil *et al.* 1994) with 0.44 µM BA and 0.05 µM NAA, and served as stock cultures for the experiments. Axillary shoots (1-2 mm long) from the primary emerging shoots could be excised every 4 weeks when the node explants were subcultured.

Induction and maintenance of somatic embryos

Nodes with axillary meristems were excised from shoots of 4 week old *in vitro* node cultures and placed in 9 cm Petri dishes with 20 ml basal medium supplied with 53.7 µM NAA. After 9 days explants were transferred to basal medium without NAA. Arising callus and roots were subcultured every 4 weeks. Somatic embryos emerging from the roots were selected and transferred to basal medium and subsequently subcultured every 4 weeks. For subculture, the tissue was filtered in order to obtain late stage elongated somatic embryos, sized 800-1600 µm.

All cultures were maintained at 27 °C in a 16-h photoperiod 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by a mixture of two types of fluorescent tubes: 60% Mazdafluor TFRS 40/Bi and 40% Philips TLM 33RS.

Conversion, and transfer to the field

Isolated elongated stage embryos (800 - 1600 µm) were placed on a filter paper (Whatman n° 3) overlaid on the basal medium in 9 cm Petri dishes. Germinating embryos were isolated and cultured in 150 ml transparent plastic containers each with 20 ml basal medium for 4-8 weeks before transfer to the greenhouse. Plants were transferred to the field in May-June after 6-12 months in the greenhouse.

Scanning microscopy

Fresh plant tissues were frozen in liquid nitrogen and inserted directly in the microscope (Phillips 625 M)

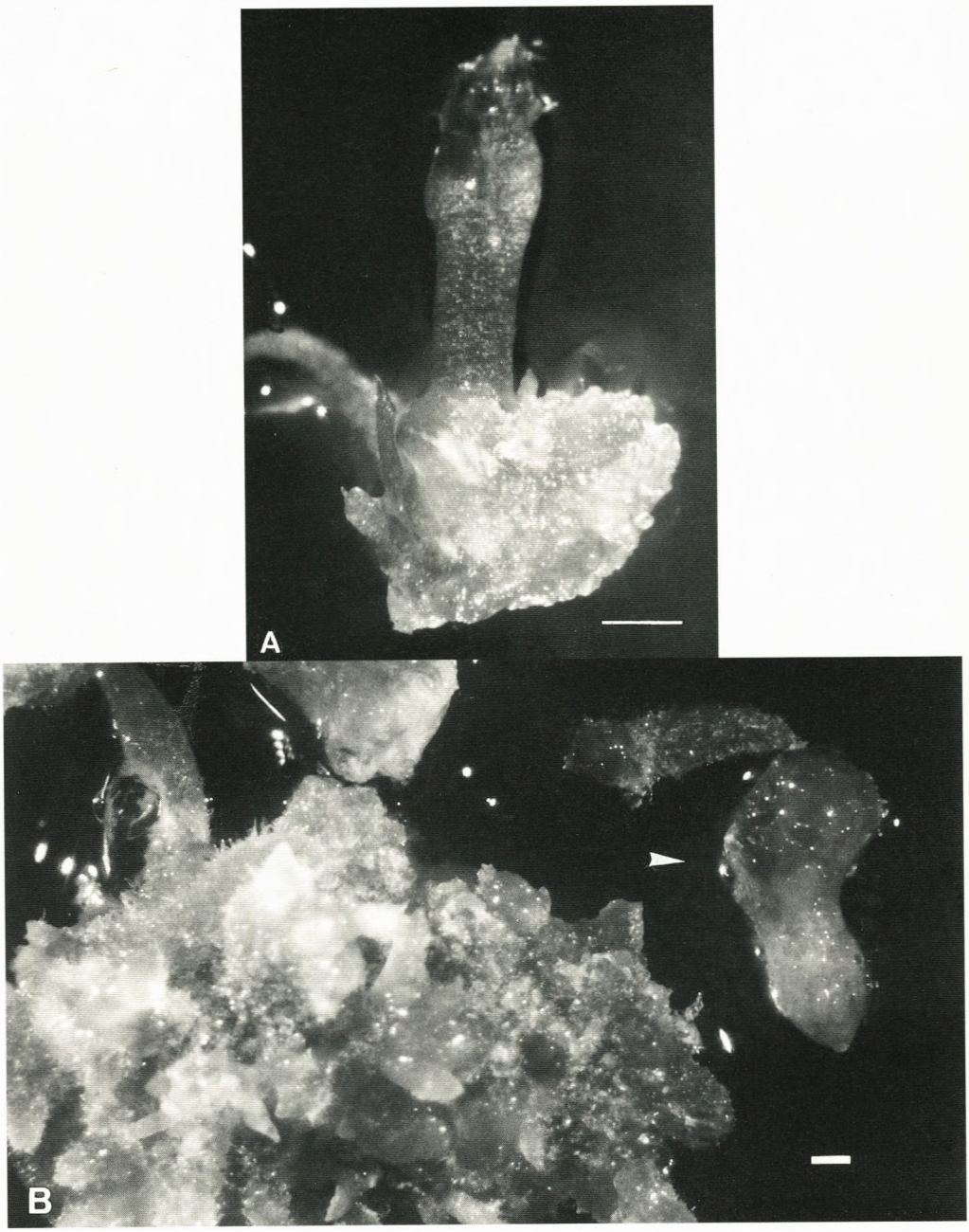
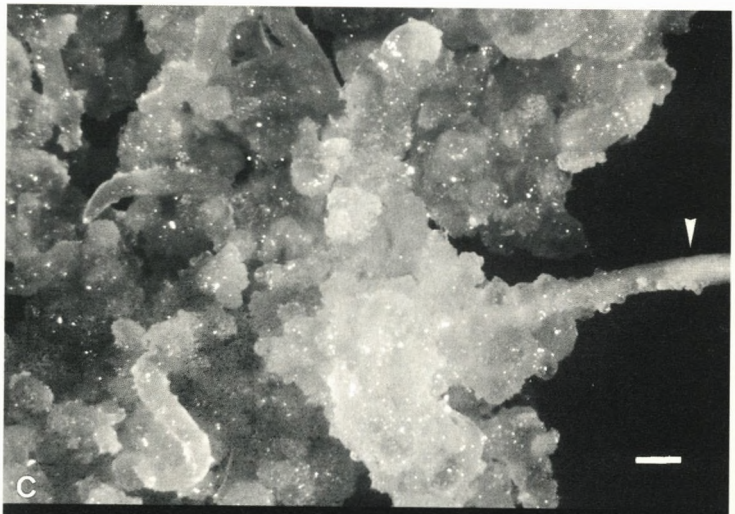


Figure 1. A-E (This and the following page). Induction of somatic embryos from axillary meristems.

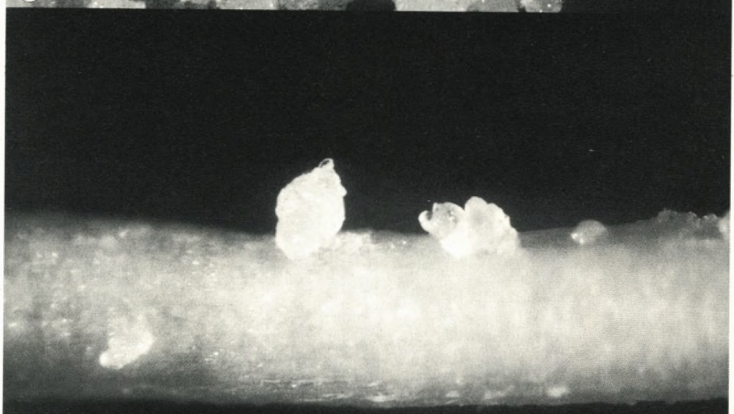
A. Basal callus on elongating axillary shoot after 9 days on 53.7 μM NAA (bar = 1 mm).

B. Callus with short root-like structures and well developed embryo (arrowhead) after 8 weeks on basal medium (bar = 1 mm).

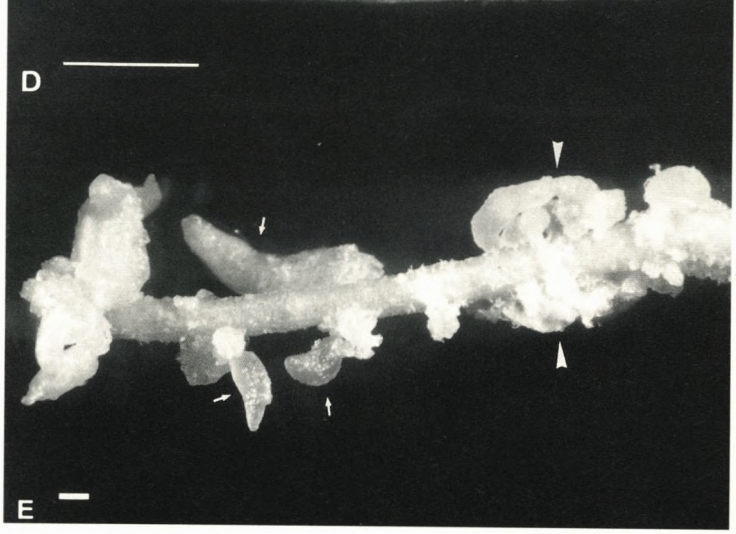
1 C. Root like structures in various developmental stages and an emerging root (arrowdead) after 12 - 16 weeks without growth regulators. The central callus necrosed. (bar = 1 mm)



1 D. Globular embryos arising from the surface of an elongated root after 12 - 16 weeks without growth regulators (bar = 1 mm).



1 E. Green elongated embryos (arrows) and clusters of growing somatic embryos (arrowhead) emerging on the surface of an elongated root (bar = 1 mm).



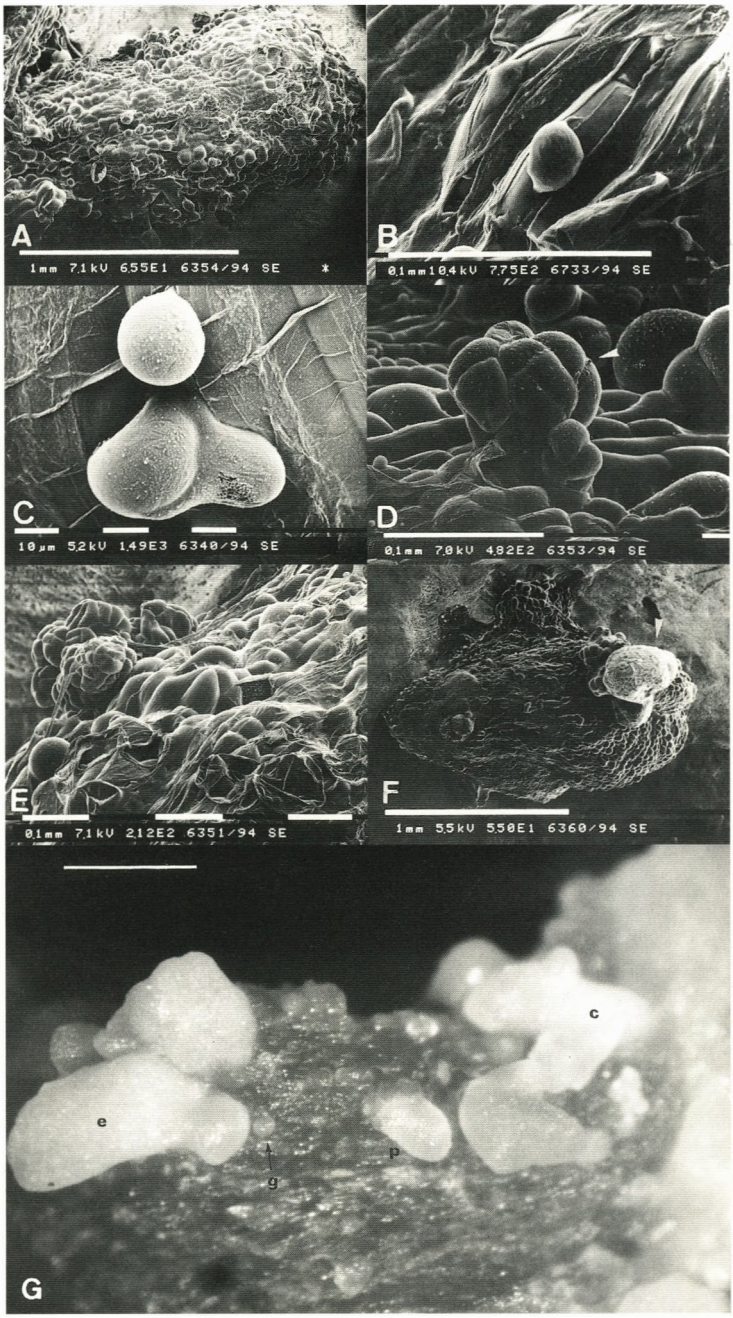


Figure 2. A-G Recurrent embryogenesis. Secondary somatic embryos (arrow) emerging through the epidermis of a primary somatic embryo.
A General view of the surface of a primary somatic embryo. B First cell of a secondary embryo emerges through the epidermis. C The first cell has enlarged and divided. D Embryo proper (arrow) sitting on suspensor-like structure. E Group of early stage somatic embryos. F General view of the development of a secondary embryo (arrow). G Macroscopic view of various stages of secondary embryo development. Time between A and G was 15 days. G = globular embryo, p = post globular stage, e = elongated (embryo) stage and c = clusters of somatic embryos. A-F: scanning microscope observations, size of bar indicated on each photo. G: bar = 1 mm.

where they were sputter-coated with gold. Pictures were taken at 5.2 - 10.4 kv on Kodak PXP 6057 film.

Results and discussion

Induction of somatic embryos

After one week on NAA containing medium primary green callus had initiated at the base of the original axillary shoots (Fig. 1A). During the first 4 weeks on auxin-free medium the callus grew and became hard and dark-green. After 4 additional weeks without auxin the tissue had evolved into a mixture of globular, friable light-green callus and small 2-4 mm elongated structures which resembled roots (Fig. 1B). At this stage a few somatic embryos could be found on some of these calluses, but this was rare. After 12 weeks without auxin the primary callus was necrotic and the roots had elongated to 10-20 mm, some with new callus at the distal portion of the root tip (Fig. 1C). Four weeks later, most of these roots were necrotic, but in a few cases individual somatic embryos or clusters of somatic embryos were observed arising on the surface (Fig. 1D and E). The somatic embryos emerged irregularly all along the roots. The process is summarised in Table 1.

Smith and Krikorian (1991) observed in *Hemerocallis* root-like structures during the transition from growth regulator containing medium to growth regulator free medium. Somatic embryos were isolated after 24 weeks on medium without growth regulators, but did not develop directly from the roots.

Table 1. The successive development of the explants prior to the emergence of embryogenic lines with asparagus Aarslev genotypes. Initially 9 days on media with 53.7 µM NAA; explants were subcultured every 4 weeks on auxin free basal media.

Weeks on auxin free media	Observation
0	Elongated axillary shoot, hard callus at basal end.
4	Hard green callus.
8	Friable callus with short roots and (rarely) normal embryos.
12	Necrotic callus, elongated roots, callus at the proximal end of roots.
16	Somatic embryos arising on the surface of necrotic roots.

In our case, each of the individual originating embryos, or clusters could be isolated and maintained as a distinct line of somatic embryos, which grew by secondary embryogenesis. Morphologically evidence for secondary embryogenesis can be seen on Fig. 1D and E where clusters of crowded embryos emerge from very restricted superficial areas of the root, and proliferate by secondary somatic embryogenesis. When mature elongated embryos, as in Fig. 1E, were subcultured on basal medium they produced quantities of secondary embryos by the process which is described in Fig. 2. A few embryogenic cells emerge through the epidermis (Figs. 2A and 2B) of the primary embryo, and divide (Fig. 2C) to produce clusters of embryos in their early stage of development (Fig. 2D and 2E). Some of these globular embryos will continue the development (Fig. 2F) and eventually

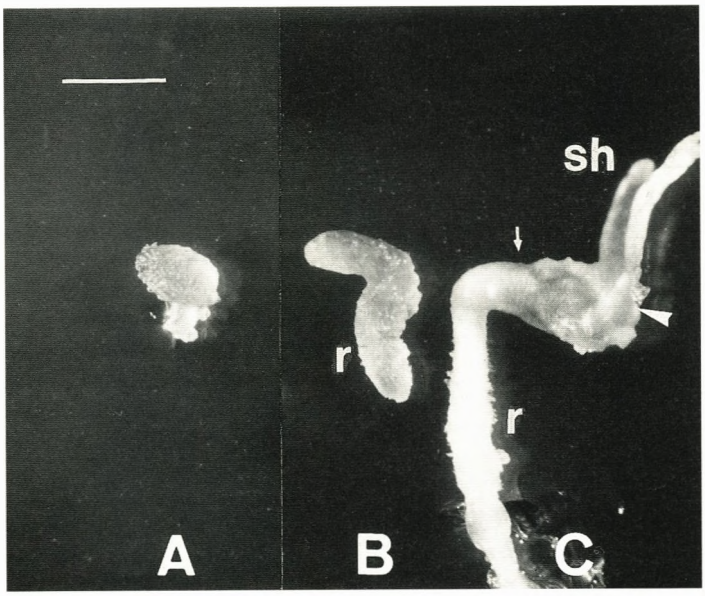


Figure 3. A-C Germinating *Asparagus* somatic embryos and plantlet developed from somatic embryo. A Elongated embryo. B Root emergence. C Shoot emergence. Cotyledon (arrow), cotylodonary sheath (arrowhead), root (r), first shoot (sh). Bar = 5 mm.

repeat the process again if the culture medium is not exhausted (Fig. 2G).

Thirty percent of the original explants produced at least one line of somatic embryos. One line of somatic embryos from each selected plant has been maintained for more than two years on basal medium without growth regulators. As also found by Reuther (1990) and Saito *et al.* (1991) visual selection is very important in order to maintain a high growth rate and high conversion potential of the somatic embryos in the cultures. We selected late stage elongated embryos with a green cotyledon at every subculture. Delbreil *et al.* (1994) obtained in some French 'white' asparagus varieties somatic embryos already 4 weeks after end of auxin treatment; whereas, herein with the 'green' Danish genotypes, only green callus was obtained at this time. So, we extended the period without auxin to 20 weeks (5 subcultures on basal medium). In these conditions, the callus developed roots from which somatic embryos arose as described. The observed differences are probably due to differences in the genetic background of the varieties, e.g. possible differences in auxin sensibility.

Maintaining embryogenic cultures without growth regulators may reduce potential somaclonal variation induced by long-term growth regulator treatment (Rueb *et al.* 1994). However, the very ability to multiply by secondary embryogenesis without an external supply of growth regulators (a sort of habituation) may be in-

terpreted as a mutation because Delbreil & Jullien (1994) presented evidence that some embryogenic lines carried an *in vitro* induced mutation which improved the ability of asparagus tissues from whole plants to produce somatic embryos, when subjected to *in vitro* culture.
We did not compare the ability of the original selected plants to produce somatic embryos with the ability of plants regenerated from the derived somatic embryos, but regenerated plantlets (2 - 5 shoots, 2 - 5 cm high), which were ready to be transplanted to the greenhouse, often produced somatic embryos from the crown-area. This was never observed on similar sized, *in vitro* grown, stock plants derived from nodes of the parent genotypes. Whole plants originating from the isolated embryogenic lines appear to have an improved ability to form somatic embryos. The heritable character of the embryogenic ability as presented by Delbreil & Jullien (1994), however, should be tested by crossing with the corresponding 'wild type' genotype.

Growth potential and germination of somatic embryos

Single elongated embryos (1.0 -1.5 mg FW) sized 800-1600 µm when subcultured, could produce 61.7 ± 10.3 mg FW (5%, n=80) of embryogenic tissue during a 3 week culture period, (and about 40 elongated embryos). Approximately 10% of the isolated somatic embryos (800-1600 µm) produced both root and shoot after 3 weeks on a filter paper placed on basal medium (Fig. 3). Plants from all four lines have been transplanted to the field for further evaluation.

Conclusion

We report here a reliable alternative for induction of somatic embryos from *in vitro* developed roots of *Asparagus officinalis*. Growth regulator treatment is very short, but the developmental period necessary for the emergence of embryogenic lines was longer compared with previous experiments with other asparagus genotypes (Delbreil *et al.* 1994). This difference could be explained by genotype variation in auxin metabolism or sensibility, if we hypothesised that appearance of embryogenic lines need a hormone deprivation. The regenerated plants appeared to have improved ability to form somatic embryos. This could be summarised as an ability for hormone independent recurrent embryogenesis. Well developed embryos can germinate, and plants have been transplanted to the field. Induction of somatic embryos on the 2 Danish cultivars of green asparagus represents the first steps towards genetic transformation by coculture of the somatic embryos and *Agrobacterium*, as developed with some French genotypes (Delbreil *et al.* 1993).

Acknowledgements

The financial support from the Danish Directorate for Agricultural Development is gratefully acknowledged. The authors thank A. M. Jaunet from the Station de Science du Sol, INRA Versailles for scanning microscopy and Dr. K. Kristiansen, Research Group Plant Breeding and Propagation, Årslev for manuscript comments.

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Asparagus Research Newsletter (1998)

Conversion of somatic embryos of *Asparagus officinalis* L.

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Key words: ABA, cloning, desiccation, green asparagus, norflurazon, somatic embryogenesis.

Abstract

The effects of application of exogenous factors such as desiccation or growth regulator treatment, on the maturation and conversion of somatic embryos of asparagus (*Asparagus officinalis* L.) were investigated. Improved air exchange and culture on filter paper could improve the fresh weight of mature somatic embryos (elongated 1600-800 µm) as compared to embryo culture directly on the medium without air exchange. Abscisic acid (0, 5, 20, 40, 200, and 1000 µM) treatment during 24 h before desiccation during 24 h in controlled environments did not improve conversion. Embryos desiccated to 73%, 61% or 45% relative humidity did not survive, regardless of pretreatment with abscisic acid.
Norflurazon stopped chlorophyll synthesis and reduced growth of the somatic embryos. However, conversion was not effected by norflurazon.

Abbreviations: ABA - abscisic acid, FW - fresh weight, RH - relative humidity, SD - standard deviation

Introduction

Producing plants from somatic embryos of asparagus has been studied intensively because of great difficulties in the conversion process. Generally conversion of mature somatic embryos to plantlets with root and shoot has many similarities to zygotic embryo development. The zygotic embryo passes through several stages during seed development and maturation. Maturation events prepare the seed for germination and subsequent development of the mature plant. During maturation, the seed increases in volume and mass due to cell expansion and reserve accumulation. Embryo desiccation occurs naturally in most seeds, and has a role in the developmental transition between maturation and germination (Thomas 1993). Desiccation tolerance can be improved by e.g. temperature, proline, abscisic acid (ABA), or osmotic treatment

(Skriver & Mundy 1990). Improved conversion of somatic embryos has been reported by various desiccation treatments including osmotic treatment by polyethylene glycol (Attree *et al.* 1991), increased carbohydrate level (Levi & Sink 1990 and 1992) and improved air exchange (Saito *et al.* 1991).

Abscisic acid levels reach a maximum during the later half of zygotic embryo development, suppressing precocious germination and modulating gene expression. Late embryogenesis abundant (lea) genes, whose expression may coincide with the rise in endogenous seed ABA level, have been described from various species (Skriver & Mundy 1990; Thomas 1993).

The influence of ABA on germination of somatic embryos of asparagus was tested by treatment with ABA (Li & Wolyn 1995), but ABA antagonists or ABA synthesis inhibitors (e.g. norflurazon) could indirectly also show whether ABA plays a role in somatic embryo development of asparagus.

This study examined parameters related to desiccation, ABA and norflurazon treatment in the conversion process of asparagus somatic embryos.

Materials and methods

Somatic embryos

Somatic embryos were isolated from roots (Ørnstrup & Jullien 1997), and maintained on agar solidified (7 g/L) basal medium without growth regulators (Delbreil *et al.* 1994). Four weeks old embryo cultures were filtered and the 1600-800 µm fraction (elongated stage) was used in the conversion experiments. General culture conditions as described by Ørnstrup & Jullien (1997).

Improved air exchange and filter paper

Somatic embryos (50 mg FW) were cultured in Petri dishes, either directly on the basal medium or on a filter paper (Whatman no 3) placed on the medium. The Petri dishes were either without Parafilm ('open') or closed by two layers of Parafilm ('closed'). Results were scored after 21 days, from one experiment with 4 replications.

ABA and controlled desiccation

Tissue was filtrated after 4 weeks on basal medium, and the 1600-800 µm fraction was cultured 24 hours in liquid basal medium, in order to moisten all tissue equally. After centrifugation the tissue was cultivated during 24 hours in liquid basal medium supplied with various levels of ABA: 0, 5, 20, and 40 µM ABA (experiment 1) and 0, 40, 200, and 1000 µM ABA (experiment 2). The cultures were centrifuged after 24 h and the tissue was blotted on filterpaper before each ABA treatment was divided in fractions for the desiccation treatments.

Desiccation was performed by placing the tissue in 55 mm Petri dishes placed inside 1 L transparent glass containers supplied with appropriate salts to reach the desired RH levels. The desiccation treatments included: "Normal treatment" (7 ml agar solidified basal medium in Petri dishes closed by 3 layers of Parafilm, placed in container without salts); "100%" RH (closed by Parafilm but

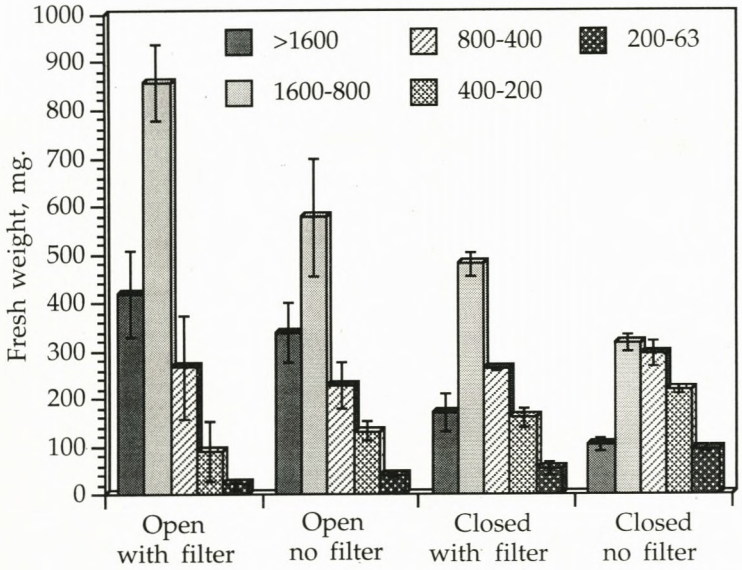

Fig. 1. Somatic embryos (1600-800 µm); 50 mg/Petri dish) were cultured for 21 days in Petri dishes with Parafilm ('closed') or without Parafilm ('open') either directly on agar solidified medium or on a filter paper placed on the agar. The obtained tissue was fractionated, and fresh weight was estimated. Bars represent SD.

without medium, placed in container without salts); 73% RH (no Parafilm, no medium, in container with 80 g NaCl + 9 ml water); 61% RH (no Parafilm, no medium, in container with 80 g NH_4NO_3 + 7 ml water); 45% RH (no Parafilm, no medium, in container with 80 g K_2CO_3 + 16 ml water). The desiccation containers were placed in the growth chamber at 27°C, and after 24 hours the embryos were transferred to 55 mm Petri dishes with 7 ml agar solidified basal medium; 5 embryos/Petri dish; 5 replications. Results were scored after 42 days.

Norflurazon

Somatic embryos were cultured in Petri dishes directly on basal medium supplied with either 0 or 100 µM norflurazon. The treatment lasted for 1, 2, or 21 days. After treatment the embryos were transferred to basal medium without growth regulators. Results were obtained from one experiment with 4 replications (10 embryos per Petri dish totalling 40 embryos per treatment). Results were scored 21 days after initiation.

Fig. 2. Effect of filterpaper on growth and maturation of somatic embryos of asparagus after 10 weeks culture. Bar = 10 mm.

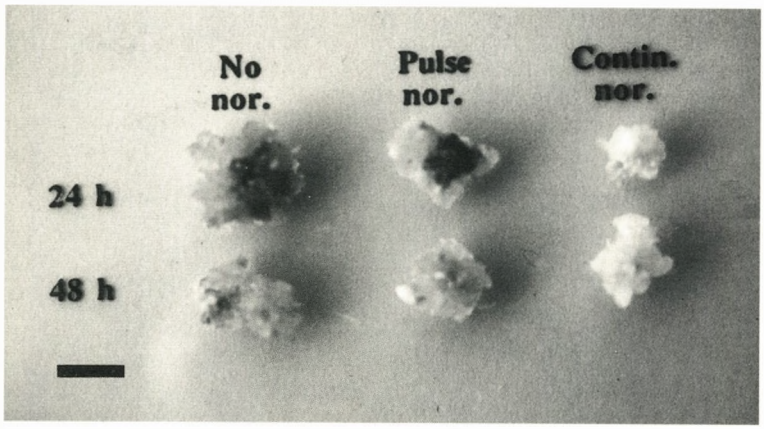

Fig. 3. Evolution of single embryos (1600-800 µm) after 21 days directly on medium. "No nor.": Non-treated embryos which were transferred to fresh medium after 24 or 48 h. "Pulse nor.": Embryos were treated with 100 µM norflurazon for 24 or 48 h. "Contin. nor.": Embryos continuously submitted to 100 µM norflurazon for 21 days, but transferred to fresh medium after 24 or 48 h. See text for details. Bar = 5 mm.

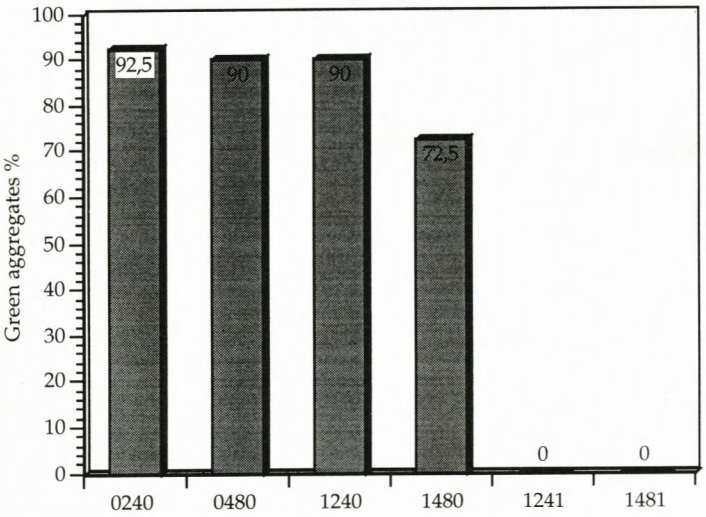

Fig. 4. Effect of norflurazon on chlorophyll synthesis. Each initial embryo formed an aggregate of new embryos during the 21 days culture period. Treatments are represented by a 4 digit code. First digit indicates initial level of norflurazon 0 = 0 µM, 1 = 100 µM. Second and third digits indicate duration of initial treatment 24 = 24 h, 48 = 48 h. The fourth digit indicates final level of norflurazon 0 = 0 µM, 1 = 100 µM. So e.g. 1480 indicates that the embryos were cultivated on a medium with 100 µM norflurazon for 48 h and then moved to new medium without norflurazon. Four replications (Petridishes) with each 10 embryos; so in total 40 embryos per treatment.

Results and discussion

Culture without Parafilm increased the fresh weight and the fraction of large (1600-800 µm) embryos (Fig. 1).
The fraction 1600-800 µm is especially interesting because it includes the elongated (mature) embryos which are able to germinate when isolated. Reduced relative humidity in the Petri dishes without Parafilm may account for some of the effects of improved air exchange. Actually the effect of improved air exchange is difficult to evaluate because it influences all components in the container atmosphere e.g. O_2, CO_2, and ethylene. Saito et al. (1991) improved conversion of somatic embryos of asparagus using a ventilative filter to close their containers. The RH was not affected by this filter, but the water content of the somatic embryos was reduced from 92.1% to 85.3%; conversion was further increased by culture on media solidified by 1% gelrite, as compared to the normal 0.2% gelrite. High levels of gelificant may reduce water disponibility in the media, and vapour pressure in the atmosphere above. Placing a piece of (filter) paper between the embryos and the medium may be another way to reduce water disponibility to the embryos.

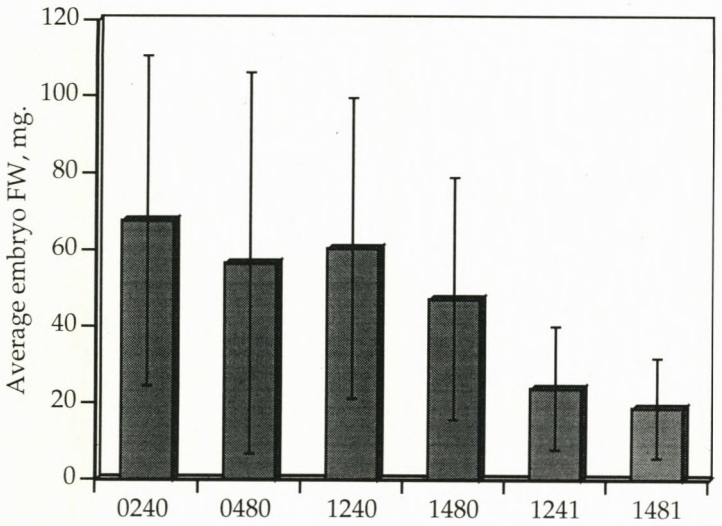

Fig. 5. Produced tissue (FW, mg) per embryo, 21 days after start of treatment. n = 40. For codes see Fig. 4. Bars represents SD.

Our results showed that culture on filter paper was able to increase the FW proportion of the tissue fraction 1600-800 µm and reduce the FW proportion of the smallest fraction 200-63 µm as compared to culture directly on the medium (Fig. 1). In our conditions secondary embryogenesis was reduced on filter paper and maturation of the somatic embryos increased (Fig. 2). Effects of improved air exchange and filter paper were additive (Fig. 1). Improved air exchange as well as culture on filter paper can be considered as a sort of mild desiccation. Desiccation or ABA treatment of well developed somatic embryos has in several cases improved the conversion rate of embryos of monocotyledons e.g. Brown *et al.* 1989 (wheat); Li & Wolyn 1995 (asparagus).

We found no effect of either ABA or desiccation in controlled environments. This could, however, be explained by inappropriate experimental conditions. In gymnosperms the optimal effect of ABA and desiccation treatments is a very restricted combination (Attree *et al.* 1991). Li & Wolyn (1995) reported that ABA treatment of somatic embryos of asparagus increased the proportion of elongated embryos and improved their conversion.

No embryos survived our controlled desiccation at 45, 61 and 73% RH; regardless of ABA treatment. If embryos were subjected to very slight desiccation (closed Petri dish without medium) or none at all (closed Petri dish with medium) no effect of the ABA treatments were found on the percentage surviving desiccation, the percentage showing new growth, or the percentage of converted embryos (results not shown). The dehydration speed rather than the water content, could play an important role in the

loss of viability for the levels 45, 61 and 73% RH. Slow dehydration (-0.2% RH/h) in a transition period in the beginning of the desiccation treatment might allow the initiation of dehydration tolerance processes (Attree *et al.* 1991; Leonardi *et al.* 1995). The dehydration speed may then be increased after this transition period. The dehydration process can be slowed by encapsulation of the single somatic embryos in alginate gels as reported by Timbert *et al.*, (1996 a + b) who found a conversion rate of 73% of carrot somatic embryos after a slow (11.5 days) dehydration from 95% to 15% RH. Encapsulation of somatic embryos from *Asparagus* has only been performed for *Asparagus cooperi* and this with a conversion frequency as low as 8.3% after 90 days at 2°C (Ghosh & Sen 1994). However, further improvement of the encapsulation and desiccation techniques in asparagus is needed before encapsulation can be utilised to improve the conversion frequency of asparagus somatic embryos.

The 24 h ABA treatment of asparagus somatic embryos may activate ABA responsive genes, but may be too short to build up sufficient quantities of products giving improved desiccation tolerance. Inhibition of ABA synthesis is another way to study whether ABA influences somatic embryo development in asparagus.

We observed that continuous culture of asparagus somatic embryos on medium containing norflurazon reduced the growth (FW) and blocked the chlorophyll synthesis (Fig. 3). However, the effect of norflurazon on chlorophyll synthesis could be reversed when the treatment lasted only for 24 or 48 h (Fig. 3 and 4). The fresh weight was little affected by the 24 and 48 h norflurazon pulse, but strongly reduced by continuous norflurazon treatment (Fig. 5). Hereby indicating that norflurazon also affects the cell metabolism at other more subtle areas. As the effect of norflurazon is mediated by inhibition of carotenoid synthesis the ABA level may be reduced also. The observed growth reduction may be a reaction to alterated activity of genes normally responding to the endogene ABA levels. Several ABA-responsive genes have been isolated (Skriver & Mundy (1990) and their references), and it is clear from the literature that ABA plays an essential role in managing the fine equilibrium within the cell, especially during osmotic stress and embryo development.

In conclusion we have shown that desiccation as represented by improved air exchange and culture on filter paper can improve the percentage of large (mature) somatic embryos as compared to embryo culture directly on the medium without air exchange. Further we have shown that norflurazon stops chlorophyll synthesis and reduces growth of somatic embryos of asparagus. We found no direct effect of ABA, however, as norflurazon indirectly shows that ABA may play a role in the development of asparagus somatic embryos. New experiments involving ABA may be able to indicate this directly.

Acknowledgements

The financial support from the Danish Directorate for Agricultural Development is gratefully acknowledged. The authors thank Dr. K.

Kristiansen, Research Group Plant Breeding and Propagation, Årslev for manuscript comments.

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Asparagus Research Newsletter (1998)

Cloning of green asparagus by *in vitro* culture.

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Key words: Ancymidol, *Asparagus officinalis*, multiplication, selection, somatic embryogenesis.

Abstract

Cloning of selected elite plants of green *Asparagus officinalis* L. genotypes (Årslev 136 and 270) has been initiated in Denmark. Elite plants were selected by evaluation of individual field grown plants for several characters including: overall yield, number of first class spears, colour, earliness and firmness. Cloning was performed by node culture and somatic embryogenesis. Potential multiplication capacity is very high in both techniques. From initially 14 nodes could be harvested about 3500 new nodes after 11 weeks. The multiplication factor for the somatic embryos was between 30 and 300 every 4 weeks. Plants regenerated by both techniques have been transplanted to the field where their performances will be followed the next years.

Abbreviations: NAA - naphthaleneacetic acid, SD - standard deviation

Introduction

Commercial asparagus (*Asparagus officinalis* L.) production in Denmark has the last years declined from 1670 ha in 1964 to less than 50 ha in 1993. Due to low yield, high wages and cheap imported asparagus. An explanation for the low production is that yield of single plants can vary as much as a 25 fold difference between the highest and lowest yielding plants, and yields as high as 13.5 t/ha has been achieved from cloned plants (Falloon *et al.* 1989).

Traditional breeding in Denmark has produced high yielding varieties (Sørensen & Thuesen 1992) but high variation in yield from single plants remain. A new selection programme in Denmark has focused on the possibilities which *in vitro* culture gives for cloning of selected plants.

Selected plants can be *in vitro* multiplied by either node culture (Yang & Clore 1973; Doré 1975; Conner *et al.* 1992; Desjardins 1992; Conner & Falloon 1993; Shigeta *at al.* 1996) or by somatic embryogenesis (Wilmar & Hellendoorn 1968; Harada 1973;

Jullien 1974; Levi & Sink 1991 and 1992; Reuther 1977; Delbreil *et al.* 1994). A review of micropropagation of asparagus can be found in Desjardins (1992). ' Somatic embryogenesis in asparagus has been reviewed by Levi & Sink (1995).
Rooting problems in the node culture has directed much research towards somatic embryogenesis. However, well growing cultures of somatic embryos are not easily established; especially the induction phase can be a difficult hurdle. But also development of root and shoot from the somatic embryos is difficult to manage. However, surmounting these difficulties will be highly rewarded by the enormous multiplication potential in somatic embryogenesis.
Here we report how we cloned selected high yielding plants by node culture and somatic embryogenesis.

Materials and methods

Plant material

Plants were selected from a three year old plantation of the two green varieties: Aarslev no 136 and no 270 (Sørensen & Thuesen 1992). Each plant was evaluated for several characters (Sørensen 1996). Four remarkable male plants were used as "mother plants" for start of the *in vitro* culture.

In vitro node cultures

Shoot producing cultures were started from nodes of secondary branches of selected field grown plants. These node cultures were maintained on a basal medium (Delbreil *et al.* 1994) with 0.44 µM BA and 0.05 µM NAA, and served as stock cultures for the further work. Cultures were maintained at 27 °C in a 16-h photoperiod with 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by a mixture of two types of fluorescent tubes: 60% Mazdafluor TFRS 40/Bi and 40% Philips TLM 33RS. Nodes from the emerging axially shoots could be excised every 4 weeks, and served as explants for new cultures. Old nodes from which the shoots had been harvested were returned to culture. Subsequently they developed shoot clusters and rhizomes.

Rooting of shoot clusters originating from nodes

Several treatments were tried in order to improve the rooting frequence of shoot clusters originating from the nodes. Nodes cultivated 4, 8 or 12 weeks on shoot medium were transferred to media with sucrose levels of 5, 7, and 9% combined with 0, 16, and 54 µM NAA. Media with 6% sucrose was combined with 16 µM NAA or 0.39 µM ancymidol. Results were scored after 100 days. Treatments included 8-20 nodes per container, with 2-6 replications. Data is presented as mean ± SD.

Induction of somatic embryos

Somatic embryos were induced from shoots of 4 weeks old *in vitro* node cultures. Excised axially meristems were placed in Petri dishes with basal medium supplied with 54 µM NAA. After 9 days the explants were transferred to medium without growth regulators. Somatic embryos were isolated from emerging roots, and maintained without growth regulators (Ørnstrup & Jullien 1997a).

Germination of somatic embryos and transfer to the field

Isolated elongated embryos (1600 - 800 µm) were plated on a filter paper (Whatman no 3) on basal medium in Petri dishes. Germinating embryos were isolated and cultured in 150 ml transparent plastic containers during 4-8 weeks before transfer to the greenhouse. Plants from both node culture and somatic embryos were transferred to the field in May-June after 6-12 months in the greenhouse.

Results and discussion

Node cultures

Axillary shoots developed from the nodes on the shoot medium. These axillary shoots could yield new nodes for further multiplication. From initially 14 nodes could be harvested about 3500 new nodes after 11 weeks (Fig. 1). Theoretically these 14 nodes can yield about 10^{12} nodes during one year.

After decapitating the newly developed shoots the old nodes were returned to culture. Subsequently they developed rhizomes at the basis of the shoot clusters. Neoformed rhizomes of asparagus do not root well (Yang & Clore 1973; Desjardins *et al.* 1987; Khunachak *et al.* 1987). However, Shigeta *at al.* (1996) reported 74% of the rhizomes forming roots after 10 weeks on rooting medium containing gellan gum (8 g/l) and glucose (5%). In nature asparagus develops both thin absorbing roots and thick storage roots. Formation of storage roots *in vitro* before transplantation can greatly improve the survival of the

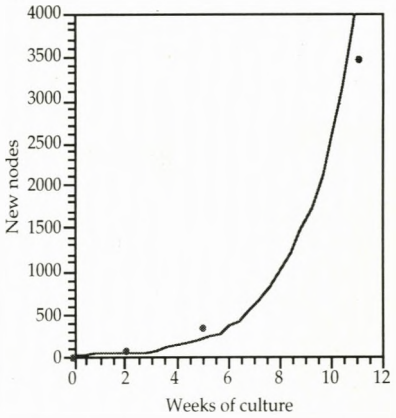

Fig. 1. Number of new nodes harvested during the first 11 weeks of culture. Number of new nodes = 20,11 exp(0,489 * weeks); R^2 = 0,97.

transplanted asparagus plants (Conner *et al.* 1992). In vitro development of storage roots can be increased by culture on medium containing 6% sucrose, 0.054 µM NAA and 0.39 µM ancymidol (Conner *et al.* 1992). However, 6 and 7% sucrose without growth regulators was sufficient for promoting of rooting in several asparagus clones (Desjardins *et al.* 1987; Conner *et al.* 1993). It may be the osmotic effect of the high-sucrose medium which triggers the initiation of roots, and the nutritional effects of the high sucrose concentration that stimulate the development of the storage roots (Desjardins *et al.* 1987; Conner *et al.* 1993). This hypothesis is based on results showing that several non- or poorly-metabolised carbohydrates supplied at equimolar levels were only able to initiate short, thin and poorly developed roots. Well developed storage roots could only form on 6 and 7% sucrose which is easily metabolised.

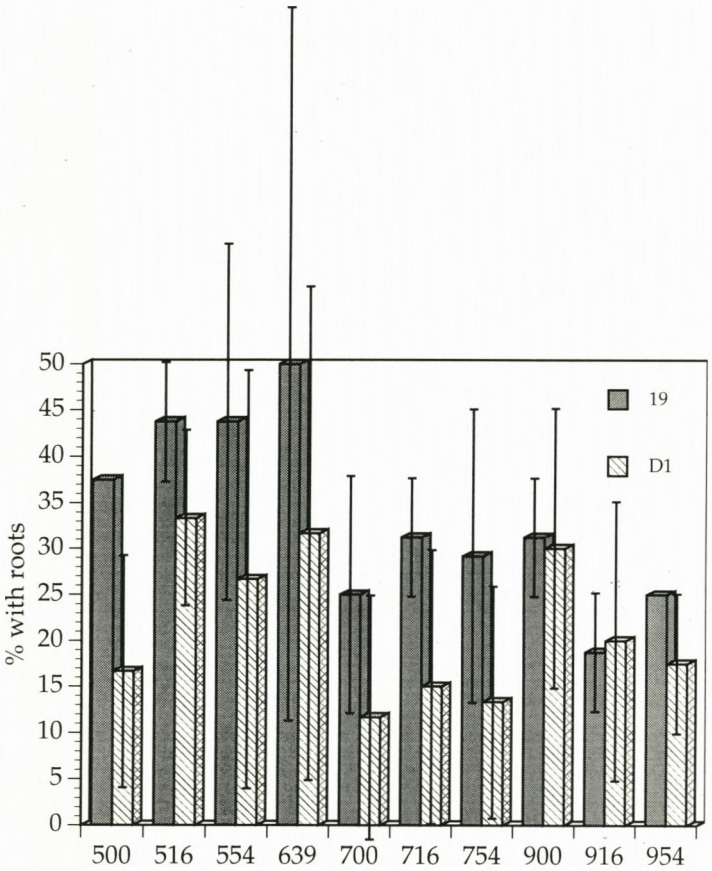

Fig. 2. Root promoting effect of some root inducing media.
The various media are represented by a 3 digit code: First digit in the code indicates the percentage of sucrose in the medium, the two next digits indicate the supplemented growth regulator concentration: 00 = no growth regulator added, 16 = 16 µM NAA, 54 = 54 µM NAA, 39 = 0.39 µM ancymidol. Figures show rooting frequency from nodes of two clones (19 and D1), subcultured 3 times on shoot inducing medium before transfer to the root inducing media. Bars indicate SD.

The root promoting effect of ancymidol and other gibberellin synthesis inhibitors is due to a reduction in the endogene level of gibberellin which has been found to be supraoptimal in asparagus node cultures (Khunachak *et al.* 1987).

We did not find that root formation from *in vitro* produced rhizomes could be increased by culture on high level (9%) sucrose medium, or on medium supplied with 6% sucrose combined with ancymidol (0.39 µM). None of our treatments showed

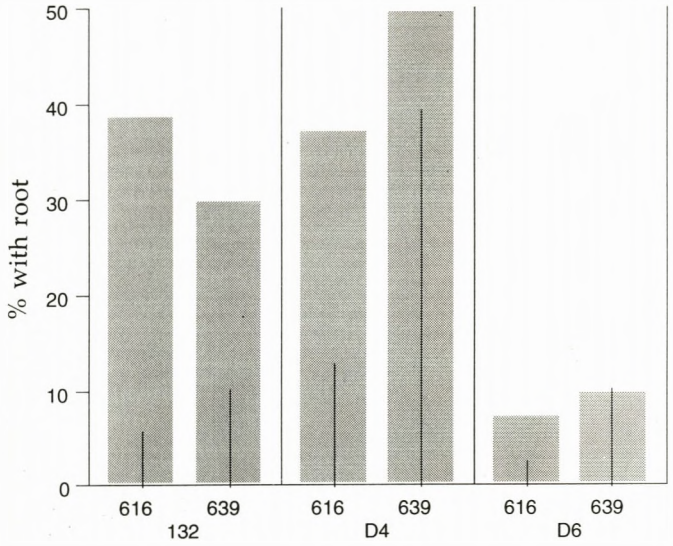

Fig. 3. The root promoting effect of two root inducing media (616 and 639) on three clones (132, D4 and D6). Lines show SD. For explanations see Fig. 2.

significant effect on rooting, however trends could be seen. Figure 2 represents results from two clones (19 and D1).

Media containing 6% sucrose supplied with 16 µM NAA or 0.39 µM ancymidol were tested for their root inducing effect on three clones (132, D4 and D6). But no significant effect were found (Fig. 3).

As no specific treatment could be selected we recommend media without growth regulators in the root induction medium (e. g. 9% saccharose), in order to avoid unwanted side effects from the growth regulators.

One of the varieties (132) showed that, shoot clusters cultivated for 12 weeks on shoot medium before transfer to rooting medium had a higher rooting percentage than shoot clusters cultivated during only 4 or 8 weeks on shoot medium. This difference was probably due to better rhizome development on the most mature shoot clusters (Fig. 4).

Induction and conversion of somatic embryos

Somatic embryos were isolated from roots and maintained on growth regulator free medium for more than two years (Ørnstrup & Jullien 1997a).

The cultures grew by secondary embryogenesis and retained their regeneration potential during the two year culture period. In conversion tests, 10% of the isolated somatic embryos produced both root and shoot after 3 weeks (Ørnstrup & Jullien 1997a). Several treatments related to desiccation or abscisic acid have been investigated (Ørnstrup & Jullien 1998) but no improvement of conversion rate has

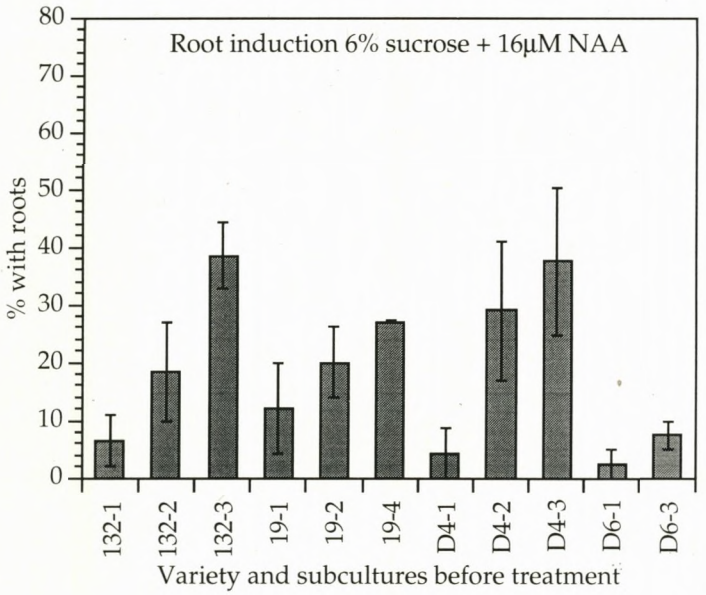

Fig. 4. Several varieties and their age/size as expressed by the number of subcultures on shoot medium (e.g. 132-3 = variety no 132 subcultured 3 times = 90 days) before transfer to root inducing medium supplied with 6% sucrose and 16 µM NAA. Bars = SD.

been found. However, one embryo can produce about $3.4*10^{18}$ new embryos per year (Ørnstrup & Jullien 1997a), so even an embryo conversion frequency of 10% can give an impressive number of plantlets.

Conclusion

We have cloned selected asparagus plants by node culture and by somatic embryogenesis. Regenerated plants have been transplanted to the field. In the next years the yield and uniformity of the cloned plants will be compared to seed propagated plants of the same varieties.

Acknowledgements

The financial support from the Danish Directorate for Agricultural Development is gratefully acknowledged. The authors thank Dr. K. Kristiansen, Research Group Plant Breeding and Propagation, Årslev for manuscript comments.

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Plant Cell Reports (1998)

Transformation of *Asparagus officinalis* using a supervirulent strain of *Agrobacterium tumefaciens*.

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Summary

Asparagus officinalis was transformed by *Agrobacterium tumefaciens*. The best result was found using a supervirulent strain (Agl1) constitutively expressing the virulence genes. This supports the hypothesis that exudates from monocot cells only at a very limited level are able to activate the virulence genes in standard str66

Key words: GUS, NPT II, PAT, somatic embryos.

Introduction

The most commonly used method for transformation of monocotyledonous plants is the bioballistic process (Christou, 1996). However, protocols for transformation of monocotyledonous plants by *Agrobacterium* have been established in a few cases e.g. in asparagus (Delbreil *et al.*, 1993), rice (Hiei *et al.*, 1994), and maize (Ishida *et al.*, 1996). *Asparagus officinalis* L. (Liliaceae) has been transformed by several procedures, including *A. tumefaciens* [Hernalsteens *et al.* (1984); Butebier *et al.* (1987); Conner *et al.* (1990)].

Somatic embryos from *Asparagus officinalis* can be induced *in vitro* from several explants sources: hypocotyls (Wilmar and Hellendoorn, 1968), stems (Reuther, 1977), cladophylls (Harada, 1973) and cladophyll cell cultures (Jullien, 1974). In some cases long-term embryogenic calluses can be obtained (Jullien, 1974; Delbreil *et al.*, 1994). Delbreil *et al.* (1993) found that cylindrical embryos (800-1600 µm) could be transformed by *A. tumefaciens* (C58 pGV2260-35SGus-int); globular embryos (200-400µm) were assayed also but no transformants were found.

The particle gun has been used as an alternative to *Agrobacterium*-mediated transformation of asparagus (Perri *et al.*, 1994). *Asparagus* protoplasts have been transformed by electroporation (Mukhopadhyay and Desjardins, 1994), but plant regeneration was not reported. So only *Agrobacterium* mediated transformed *Asparagus* tissue has been able to regenerate plants.

The experiments reported here were performed in order to improve previous results on transformation of *Asparagus officinalis* by

Agrobacterium tumefaciens (Delbreil *et al.* 1993).

Materials and methods

Agrobacterium strains:
Transformation was carried out with two combinations of vector and *Agrobacterium tumefaciens* : C58 GV 2260-Gus-int (GV2260) (Delbreil *et al.*, 1993); and Agl1 pBPGi (supervirulent, constitutively expressing the virulence genes), constructed by the authors.

Most of the work was done with *A. tumefaciens* strain C58 GV 2260 which contains a 35S Gus-int and a nos-npt II gene (Deblaere *et al.*, 1985; Vancanneyt *et al.*, 1990).

Construction of transformation vectors:
The plasmid pBPGi was constructed by standard recombinant techniques (Maniatis *et al.*, 1982) (Fig 1). The plasmid p35SAc (derivative of pUC 18 with the *bar* gene introduced; a gift from Dr. P. Eckes, Hoechst, Germany) was cut by *Eco* RI and the resulting fragment containing the *bar*gene between the 35S promotor and the 35S terminator was ligated into the *Eco* RI digested binary vector pBin 19 (Bevan, 1984; Bevan and Chilton, 1982) which already contained an *npt* II gene controlled by the *nos* promoter and terminator (see figure 1). The resultant intermediate plasmid (pBIPAT) was opened by *Hind* III and a fragment obtained from *Hind* III digestion of p35S-Gus-int [with an intron containing *uid*A gene controlled by the 35S promotor and the 35S terminator, (Ohta *et al.*, 1990)] was ligated in the pBIPAT. This new binary vector is referred to as pBPGi. pBPGi was mobilised into the super virulent strain *A. tumefaciens* Agl1 using the triparental mating system (Drapper *et al.*, 1988). The new strain is referred to as Agl1 pBPGi.

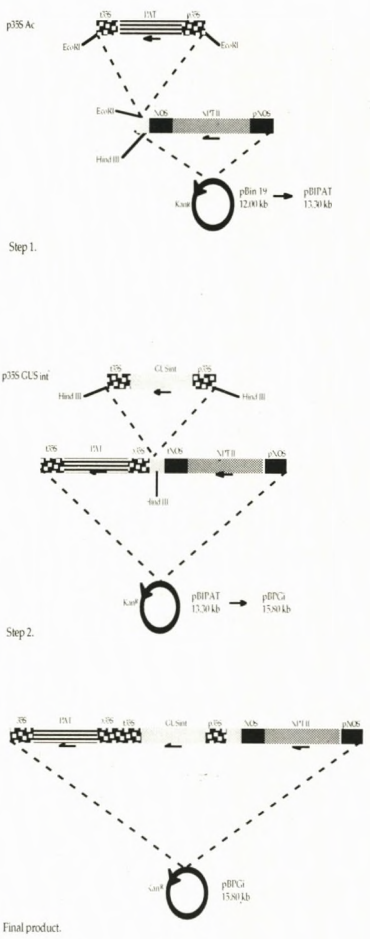

Figure 1. The two steps involved in the construction of the plasmid pBPGi, which combines the gene for kanamycin resistance (*npt* II), the gene for Basta resistance (*bar*) and the ß-glucuronidase gene (*uid*A) containing an intron. The orientations of the *bar* and *uid*A genes were deduced after enzymatic digestion. See text for details.

Plant material

Somatic embryo culture:

Two embrogenic lines were used: Line A (S 81 A.2) isolated from a male line (F_1 hybrid 81 A) provided by INRA, Versailles and line B (S 8.1) isolated from a male plant provided by Jacques Marionnet GFA. Habituated embryogenic lines of *Asparagus officinalis* were obtained according to Delbreil *et al.*, 1994. These habituated embryogenic lines were subcultured every 3-4 weeks on MSN-medium (Delbreil *et al.*, 1994) in a growth chamber 25 °C, 70 % relative humidity and 16h/day fluorescent light (40-70 $\mu Em^{-2}s^{-1}$).

Whole plants are normally obtained by transferring root and shoot forming embryos to a new medium without growth regulators, and later transplanting the plantlets to the soil under greenhouse conditions.

Fractionation of embryos:

The embryogenic tissue was fractionated by washing with liquid MSN through a series of filters with the following mesh sizes: 63, 125, 200, 400, 800, and 1600 µm. For most of the experiments was only used the fraction retained on the 800 µm mesh filter (elongated embryos).

Bacteria:

Bacteria stored at 4 °C for 4-8 weeks on agar solidified LB media supplied with the appropriate antibiotica were revitalizated at 28 °C during 24 h on solid LB media supplied with the appropriate antibiotica. The day before the coculture 10 ml of liquid LB medium was inoculated with bacteria and put on shaker overnight (18 h, 28 °C, 190 rpm). When the optical density at 600 nm (OD_{600}) reached 0.8 (= 10^8 bacteria per ml) the culture was centrifuged (15 min at 2000 g). and the pellet was resuspended in liquid MSN.

Coculture:

One gram of embryos was used pr 5 ml of bacteria solution. After 15-20 min coculture they were subjected to a 3 x 3 min vacuum infiltration (-0.79 bar). After centrifugation (4 min at 840 rpm ≈ 140 g) the bacteria solution was pipetted off and the embryos were blotted dry before transfer to paper filters (Whatman no 3, 7 cm in diameter) in Petri dishes containing 20 ml agar solidified MSN-medium (50 or 500 mg embryos per Petridish). In some cases the blotted embryos were suspended in liquid medium (50 mg/ml) and one ml was transferred to each Petridish/paper filter. To test the effect of acetosyringone it was added to the coculture medium (MSN) to give final concentrations of 0, 2.5, 10 and 100 µM. Experiments were performed with at least two replications (frequently with 3 or 4 replications).

Selection of transformants:

The coculture was performed in the dark at 27 °C for 48 or 72 h. The filter papers with the embryos were then transferred to medium containing cefotaxime (400 mg/L) and placed in light. Generally the filter papers with the embryos were transferred to the selection medium containing kanamycin (100 mg/L) and cefotaxime (400 mg/L) at day 7 after start of coculture. Subculture to new medium for selection was performed every 21 days (day 28, 49 and 70 after start of coculture). After at least 70 days on selection medium the green and healthy looking callus/embryos were selected from the more or less brown non transformed tissue, and transferred to new kanamycin and cefotaxime con-

taining medium. Embryos surviving here were considered transformed, and subjected to further test.

GUS histochemical assay.
Histochemical GUS assay was performed either on apparently kanamycin resistant tissue or 6 days after coculture, without subjecting to selection. The procedure described by Jefferson (1987) was applied with a few modifications. Samples were incubated in a 0.5 mg/ml solution of X-Gluc (5-bromo-4-chloro-3-indolyl-B-D-glucoronide, final conc. 7 mM) in assay buffer (50 mM potassium phosphate buffer at pH 7.0 and 0.05 mM each of potassium ferricyanide and potassium ferrocyanide). Samples were submerged and subjected to 10 min vacuum infiltration (-0.79 bar) before a 24 h incubation at 37°C. Green tissue was then decolorated by 70 % ethanol during 20 min., and the number of GUS expression units (blue cells or groups of cells) were observed under a dissecting microscope (table 2).

Results and discussion

Somatic embryos of *A. officinalis* that had been cocultivated with *A. tumefaciens* AGL1 pBPGi were cultured on kanamycin containing medium, and after 2 months it was possible to isolate 6 lines of green and well growing tissue from a total of 8 Petri dishes with each 50 mg somatic embryos initially; and after a further 4 weeks it was possible to isolate further 4 lines of green tissue. Thus giving 1 line per 40 mg of original tissue. However, of these 10 lines only one survived the subculture to new kanamycin containing selective medium. So the frequency of stable transformation was

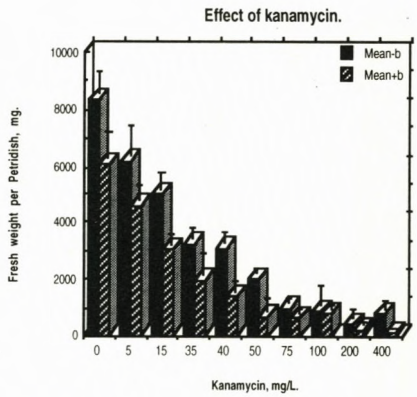

Figure 2. Growth of somatic embryos (size 1600 - 800 µm) after 70 days on the respective levels of kanamycin. Cefotaxime (400 mg/L) was included at all levels. Results are mean of three replications with bacteria (+b) or without bacteria (-b). Error bars indicate SD level. Initial FW = 50 mg.

one per 400 mg tissue (= 2.5 pr gram tissue). This line showed positive response in a GUS test.
In order to minimise the chances for a false positive GUS response, the reaction was stopped by washing the tissue in ethanol after 2 h 30 min in the GUS reaction medium. Already after one hour in the GUS reaction medium the meristematic regions for both the shoot and the root of the tested embryos was densely blue. The very few transformants found when GV2260 was used (Table 2) may be explained by low activation of the virulence genes in GV2260 as the super virulent bacterial strain Agl1 gave more transformants. The successful *Agrobacterium*-mediated transformation protocols for rice (Hiei *et al.* 1994) and maize (Ishida *et al.* 1996) included supervirulent strains of *Agrobacterium* as well. However, the choice of vectors and bacterial strains was very important (Hiei *et al.* 1994).

Table 2. Transformation events expressed in number of strongly Gus positive areas in the total of the cocultured tissue.
(-) no test performed.

Exp. no	Plasmid	Plant line	Cocultured, mg	Age at Gus test	Gus pos	Lines kanamycin resistant	Lines kan. res and Gus pos.
I	GV2260	S8.1	2400		-	0	0
II	GV2260	S8.1	2100		-	0	0
III	GV2260	S81 A.2	3000	70 days	0	0	0
IV	GV2260	S81 A.2	1500	78 days	2	0	0
V	GV2260	S81 A.2	no filtration 9500	28 days	8	0	0
VI	GV2260	S81 A.2	600	6 days	1 1	not tested	-
VII	Agl1 pBPGi	S8.1	400	70 days	1	1 0	1

Further *Agrobacterium*-mediated transformation efforts on asparagus should pay attention to this problem as the recalcitrance of monocots to transformation by *Agrobacterium* may be due to low activation of virulence genes in *Agrobacterium* by exudates from scarified cells of monocots (Baron and Zambryski, 1995). We did not scarify the tissue on purpose. However, we suppose our handling of the embryos on the steel filters did hurt the embryos sufficiently. No effects of acetosyringone were found.

Selection of transformants:
Monocotyledonous plants have in several cases shown relative high resistance to kanamycin (Schrott, 1995). In our experiments, the somatic embryos of *A. officinalis* have shown a relative high resistance to kanamycin. However, the growth of the somatic embryos was substantially reduced (figure 2); as well as chlorophyll synthesis. Non transformed tissue grown on 100 mg/L kanamycin is brown or transparent 9 weeks after coculture.

Basta appeared to be more toxic than kanamycin. All embryos died in a preliminary experiment with 6 mg/L (results not presented). With the correct level for selection Basta may be a good selective agent in transformation work with asparagus.
We found that the 400 mg/l Cefotaxime media actually improved growth of the embryos (figure 3).

Size of explants for transformation
Several authors have found that competence for transformation was restricted to a certain developmental stage of the plant cells (McKinnon *et al.*, 1996), and as Delbreil *et al.* (1993) showed that the elongated embryos in the size of 1600-800 µm was the best responding tissue, most of our work was performed with this developmental stage of the embryos.
However, when GUS staining was performed after only 7 days coculture, dark blue staining was restricted to the small globular secondary embryos on the surface of the original embryos.

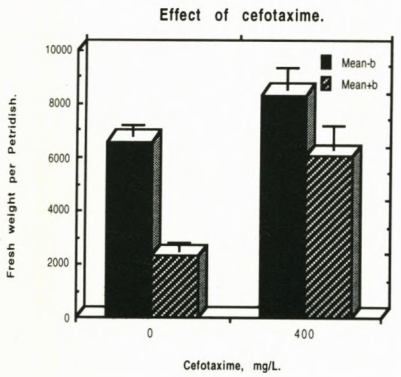

Figure 3. Effect of cefotaxime on growth of somatic embryos (size 1600 - 800 µm) after 70 days on the medium. Fresh weight (mg) is higher on cefotaxime containing medium. Results are mean of three replications with bacteria (+b) or without bacteria (-b). Error bars indicate SD. Initial FW = 50 mg.

We generally used 4 weeks old cultures which were filtered and used at once; but as culture age has been shown to be important (McKinnon *et al.*, 1996) our results may be improved if the various fractions were transferred to new medium a few days before cocultivation. Scarification a few days before coculture could also induce cell division, so, young fast dividing cells were abundant at the coculture.

As we do not present confirmation of the transformations neither by molecular biology (Southern) nor by the heritability of the introduced characters (Basta and kanamycin resistance and ß-glucuronidase activity) the presented results should only be considered preliminary. However, these confirmations are under way.

Acknowledgements

The financial support from the Danish Directorate for Agricultural Development is gratefully acknowledged. The authors thank Dr. K. Kristiansen and Kirsten Brandt, Research Group Plant Breeding and Propagation, Årslev for manuscript comments

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Plant Cell, Tissue and Organ Culture (1998).

Long term culture of *Alstroemeria x hybrida* L. somatic embryos in liquid media without growth regulators.

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Key words: Inca lily, micropropagation, ornamental, ovule, somatic embryogenesis, suspension culture.

Abstract

Somatic embryos were induced from immature zygotic embryos, contained in ovules, isolated 8 days post pollination. Induction of somatic embryos could be performed both with and without growth regulators. The somatic embryos were obtained either by neoformation on abnormal plantlets arising from the cultivated ovules, or directly from the ovule, coming probably from the germinated zygotic embryo. New somatic embryos developed by secondary embryogenesis and could be used for initiation of embryogenic cultures. These cultures were maintained for 18 months on a modified Murashige and Skoog semi solid medium without growth regulators; and in the liquid equivalent of this medium during 9 months. Secondary embryogenesis was prevailing in both liquid and solidified growth regulator-free medium. One month culture in liquid medium resulted in a 12-fold increase in fresh weight. By selecting the best developed (elongated) embryos from the cultures a high rate of conversion was obtained giving both plantlets and flowering plants.

Abbreviations: BA - 6-benzylaminopurine, DAP - days post pollination, 2,4-D - 2,4-dichlorophenoxyacetic acid, FW - fresh weight, NAA - naphthaleneacetic acid.

Introduction

Alstroemeria species are rhizomatous monocotyledonous perennials which have produced many hybrids grown for their attractive flowers with excellent vase-life. Commercial interest has formerly been restricted to cut-flower production. However, the increasing interest for producing *Alstroemeria* as pot plants makes breeding for low and uniform plants necessary, as well as an efficient method to produce quality

plantlets. Cloning of selected plants in *Alstroemeria* is at present made by rhizome division, either *in vivo* or *in vitro* (Hutchinson *et al.* 1994; Monette 1992).

The *in vitro* multiplication rate in *Alstroemeria* is determined by the number of rhizome tips on the plantlets when they are divided. Other methods for efficient multiplication e.g. rhizome induction from apical buds of vegetative stems (Ørnstrup *et al.* 1994) or by somatic embryogenesis (Gonzalez-Benito & Alderson 1990, 1992 a, b; Hutchinson *et al.* 1994; Schaik *et al.* 1996) have been investigated, but no efficient techniques for large scale propagation have yet been published.

Formerly reported induction of somatic embryos and their maintenance on agar solidified medium have used zygotic embryos as explants, especially immature zygotic embryos have proved good. Immature zygotic embryos have also been applied in the breeding of *Alstroemeria* in order to overcome interspecific hybridization barriers (Buitendijk *et al.* 1995; Jeu & Jacobsen 1995; Kristiansen 1995; Winski & Bridgen 1988).

Here we report how abnormal germinating immature zygotic embryos could induce somatic embryos that were able to multiply by secondary embryogenesis on medium without growth regulators, and in the liquid equivalent of this medium.

This experiment is part of a project with the overall aim to improve micropropagation efficiency in order to make a production of micropropagated *Alstroemeria* plantlets for pot plant production profitable.

Materials and methods

Plant material

Interspecific crosses were performed between (A): *A. presliana* and *A. ligtu* and (B): *A. pulchella* and *A. pelegrina*. Likewise self pollinated flowers from an unnamed clone of the 'Butterfly' type (tetraploid *A. Pulchella* X *A. Pelegrina)* were used (C).

Obtaining somatic embryos

Calli from cross A and B were obtained from ovules producing abnormal plantlets as described by Kristiansen (1995). Ovaries were removed 8-14 days after pollination (DAP), sterilised by dipping them in 96% ethanol and burning the ethanol afterwards. The immature seeds (ovules) were then dissected and placed on a basal medium (BM) consisting of MS-medium (Murashige & Skoog 1962) with 6% sucrose. "Germination" occurred about 7 weeks after start of culture. Then the "germinated" ovules were transferred to a basal medium (Kristiansen 1995) with 3% sucrose (BM3) and 4 weeks later calli with somatic embryos were isolated.

Ovules from self pollinated 'Butterfly' flowers were dissected 8 or 12-14 DAP as described above, and cultured on basal medium with 3, 6 or 25 % sucrose supplied with 0, 1, 20 µM BA combined with 0, 1, 20 µM 2,4-D in all combinations; or 20 µM kinetin with 20 µM NAA. After 7 weeks ovules were transferred to growth regulator free BM3 medium and subsequently subcultured on this medium every 4 weeks. Final results were scored 16 weeks after isolation of ovules. After 12 weeks a few abnormal germinating zygotic embryos had developed a

white non differentiated compact tissue (callus) from which it was possible to isolate somatic embryos. Isolated somatic embryos were maintained on BM3 (20 ml in 9 cm Petri dishes). At each subculture (every 4 weeks) a visual selection was performed, and only single embryos (globular or later stages) or tissue with early stages of embryos attached were subcultured.
Cultures were maintained at 20°C day and 15°C night in a 16-h photoperiod and a photon fluence of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by a mixture of two types of fluorescent tubes: 60% Mazdafluor TFRS 40/Bi and 40% Philips TLM 33RS.

Liquid culture and conversion of somatic embryos

A liquid culture was initiated from crossing (A), 9 months after isolation of the first somatic embryos, by transferring 400 mg embryos to 250 ml Erlenmeyer flasks with 40 ml medium. The initiated cultures were maintained in 150 ml transparent plastic containers with 20 ml liquid medium and 200 mg inoculum. A sample of 200 mg tissue consisted of either about 40 globular or elongated somatic embryos (800-1600 µm), or 3-4 aggregates of embryogenic tissue at an early stage of development. The liquid medium consisted of BM3 medium without agar. The medium was changed every 7 days, by decanting the old medium. Fresh weight (FW) was determined at each change of medium. All liquid cultures were agitated on an orbital shaker (110 rpm), under diffuse light. New cultures were initiated every 28 days by selecting embryos at defined stages (single globular, single elongated or aggregates of early-stage embryos) from older cultures in liquid medium. Culture conditions as mentioned earlier.
For conversion isolated embryos were cultured on agar solidified BM3 medium. Resulting plantlets were transplanted to sterilised sphagnum and grown until flowering in a greenhouse.

Histological observations

Embryogenic aggregates from four weeks old liquid cultures were selected at day 0 and day 28 after transfer to solid medium. Fixation was performed overnight at room temperature with 8% paraformaldehyde in a 0.1 M solution of Pipes buffer (pH 6.9) followed by a series of ethanol and histolemone® (Carlo Erba) washes. The tissue was then embedded in paraffin wax, sliced in 10 or 15 µm sections and stained with toluidine blue (0.1% aqueous solution) for a few seconds, before observation by a light microscope.

Experimental design

Genotype C experiments consisted of 5 ovules per treatment and included 3 replications for the ovule age "8 days post pollination" and 2 or 3 replications for the ovule age "12-14 days post pollination". Response from the 'Butterfly' ovules were sporadic (raw data are presented in table 1 and 2).
Growth estimations in liquid culture with starting FW = 200 mg included mean from 4 experiments with at least 4 replications in each experiment. Results with starting FW = 500 and 1000 mg included mean from one experiment with 4 replications. Exponential curve fitting was performed by DeltaGraph 2.0.2 for Macintosh.

Results

Induction of somatic embryos: effect of sugar, growth regulators and age of ovules

Ovules from self pollinated 'Butterfly' flowers were isolated a few days (8 and 12-14) post pollination and plated on different combinations of sugar and growth regulators.

The responses were scored in three classes: a) ovules producing normal plantlets b) ovules producing abnormal plantlets and c) ovules producing somatic embryos directly. Dead or non-responding ovules were not scored.

Normal plantlets grew vigorously and never produced somatic embryos. On the contrary, abnormal slow growing plantlets frequently formed an enlarged deformed cotyledon producing a characteristic sort of white compact tissue which produced somatic embryos.

The third class of ovules gave no plantlets, but produced somatic embryos directly without an intermediary callus phase. These embryos could originate from the germinated zygotic embryo.

The number of responding ovules were low and sporadic (Tab. 1 and 2).

Twenty two percent of the 450 ovules isolated 8 days post pollination were able to germinate normally; 14% produced abnormal plantlets and 10% were able to produce somatic embryos (Tab. 1).

Only 1.3% of the 320 ovules isolated 12-14 days post pollination were able to germinate normally, 2.2% produced abnormal plantlets and none produced somatic embryos (Tab. 2).

Approximately 10% of the ovules incubated on 3 or 6% sucrose developed normal plants as compared to 1.5% on a medium with 25% sucrose. Normal plantlets were found on all combinations of BA and 2,4-D.

Abnormal plantlets with a white enlarged cotyledon and somatic embryos (Fig. 1) were found on growth regulator free media and on several combinations of growth regulators with no clear trend. No ovules plated on medium supplied with kinetin 20 µM and NAA 20 µM germinated.

Abnormal plantlets developed either both the shoot and root meristem, or only the primary root meristem. They yielded often a white enlarged cotyledon with numerous somatic embryos (Fig. 1) probably originating from this cotyledon. Somatic embryos were found either on the surface of the white compact tissue, or more or less embedded in it. Some of these embryos were isolated by careful dissection from the tissue.

In some cases, the cotyledon of these abnormal plantlets developed abundantly a particular variety of callus evolving from the distal part of the cotyledon towards the meristematic part.

This callus was mainly composed of white globular units, 2-4 mm in diameter, which coloured the medium dark by excretions (Fig. 2). The callus itself was also partly covered by these black excretions. As elongated embryos could develop from this black excreting callus 2-4 weeks after subculture (on BM3 without growth regulators) we interpreted these globular units as globular embryos.

"Non germinating ovules" yielded in some cases directly somatic embryos. In this case the ovule turned dark and produced a few well developed embryos which could easily be isolated and maintained on semi solid BM3 medium.

Table 1. Development of self pollinated 'Butterfly' ovules isolated 8 days post pollination. Results are number of ovules responding. Normal plantlet = ovules producing plantlets with normal shoot and root; Abnormal plantlet = ovules germinating with white deformed cotyledon; Somatic embryos = ovules producing somatic embryos without intervening callus phase; n = 15 in all treatments; total = number of plated ovules pr growth regulator combination. Results were scored after 16 weeks. Growth regulator concentration as µM.

Response		Normal plantlets				Abnormal plantlets				Somatic embryos			
Sucrose (%)		3%	6%	25%	All	3%	6%	25%	All	3%	6%	25%	All
BA	2,4-D												
0	0	2	1	0	3/45	1	2	0	3/45	0	3	0	3/45
0	1	0	2	0	2/45	1	3	0	4/45	0	1	0	1/45
0	20	1	2	0	3/45	0	0	0	0/45	0	0	0	0/45
1	0	1	3	0	4/45	0	2	3	5/45	1	2	0	3/45
1	1	2	0	1	3/45	1	0	0	1/45	2	0	0	2/45
1	20	1	0	0	1/45	0	0	0	0/45	1	0	0	1/45
20	0	3	2	0	5/45	1	1	2	4/45	0	1	0	1/45
20	1	4	3	1	8/45	1	1	1	3/45	1	1	0	2/45
20	20	1	3	0	4/45	0	1	0	1/45	1	1	0	2/45
Kin 20	NAA 20	0	0	0	0/45	0	0	0	0/45	0	0	0	0/45
Total		15/150	16/150	2/150	33/450	5/150	10/150	6/150	21/450	6/150	9/150	0/150	15/450
%		10.0	10.7	1.3	22.0	3.3	6.7	4.0	14.0	4.0	6.0	0.0	10.0

Table 2. Development of self pollinated 'Butterfly' ovules isolated 12-14 days post pollination. Number of ex-plants varied, so results are presented as number of explants germinating or producing abnormal plantlets with an enlarged white cotyledon per number of cultured ovules. Results were scored after 16 weeks. Growth regulator concentration as µM. For abbreviations see table 1.

Response		Normal plantlets				Abnormal plantlets			
Sucrose (%)		3%	6%	25%	All	3%	6%	25%	All
BA	2,4-D								
0	0	0/15	1/15	0/10	1/40	1/15	1/15	0/10	2/40
0	1	0/10	1/15	0/10	1/35	0/10	1/15	0/10	1/35
0	20	0/10	0/15	0/10	0/35	0/10	0/15	0/10	0/35
1	0	0/15	0/10	0/10	0/35	0/15	0/10	0/10	0/35
1	1	0/10	0/10	0/10	0/30	0/10	0/10	0/10	0/30
1	20	0/10	0/15	1/10	1/35	0/10	0/15	0/10	0/35
20	0	0/10	0/15	0/10	0/35	1/10	0/15	1/10	2/35
20	1	1/20	0/15	0/10	1/45	1/20	0/15	0/10	1/45
20	20	0/10	0/10	0/10	0/30	1/10	0/10	0/10	1/30
Total		1/110	2/120	1/90	4/320	4/110	2/120	1/90	7/320
%		0.9	1.7	1.1	3.2	3.6	1.7	1.1	2.2

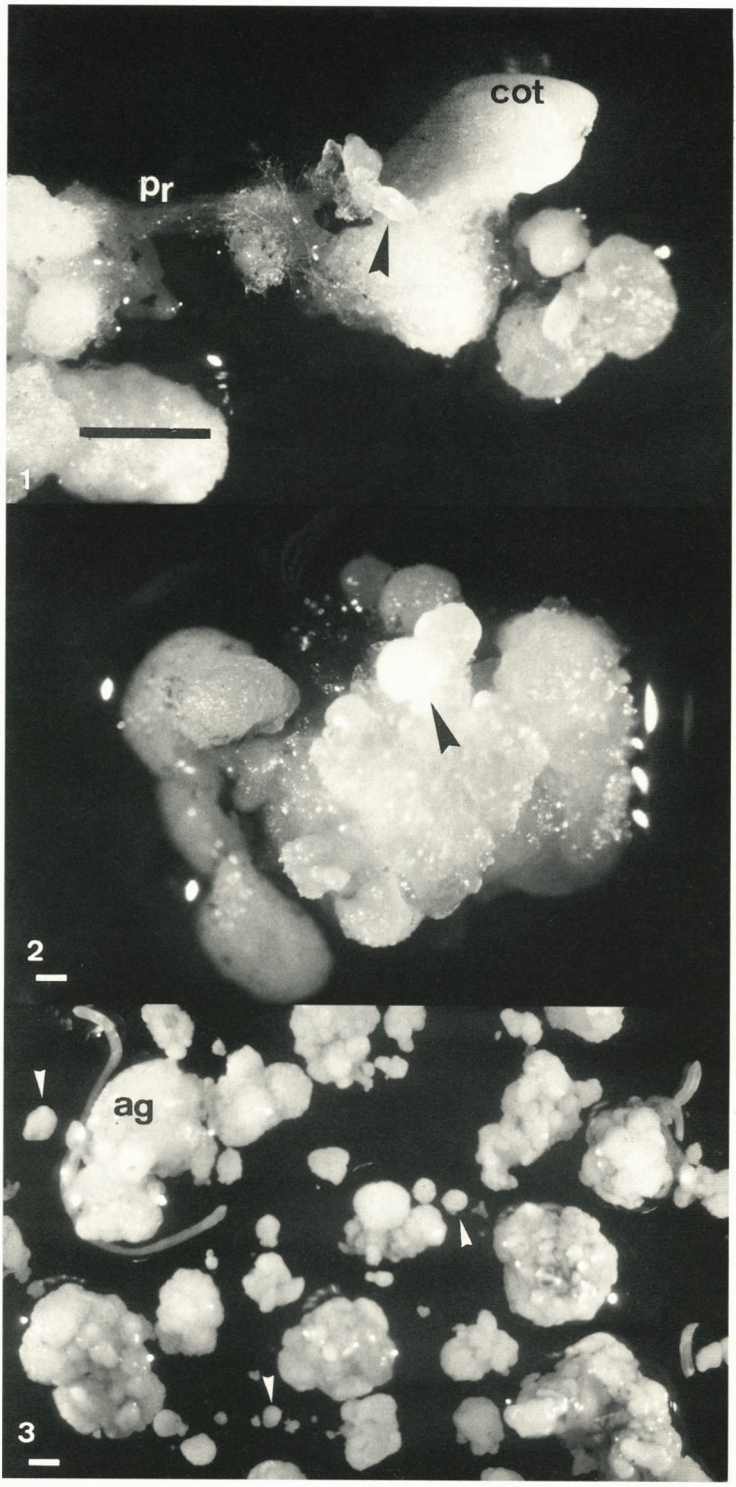

Figure 1. Abnormal *Alstroemeria* plantlet (from a 'Butterfly' ovule) germinating with a deformed cotyledon from which the first somatic embryos (arrowhead) originate after 2 weeks on BM3 medium. Cotyledon (cot), primary root (pr). Bar = 5 mm.

Figure 2. *Alstroemeria* callus from a 'Butterfly' genotype producing a characteristic black exudate. The globular structures (arrowhead) could develop lines of somatic embryos from which plantlets were regenerated. Bar = 1.2 mm

Figure 3. *Alstroemeria* interspecific crossing between *A. presliana* and *A. ligtu*. Various stages of embryogenic tissue after 28 days in liquid BM3 medium. Aggregates (ag) as well as single somatic embryos (arrowhead) are visible. Bar = 1.2 mm

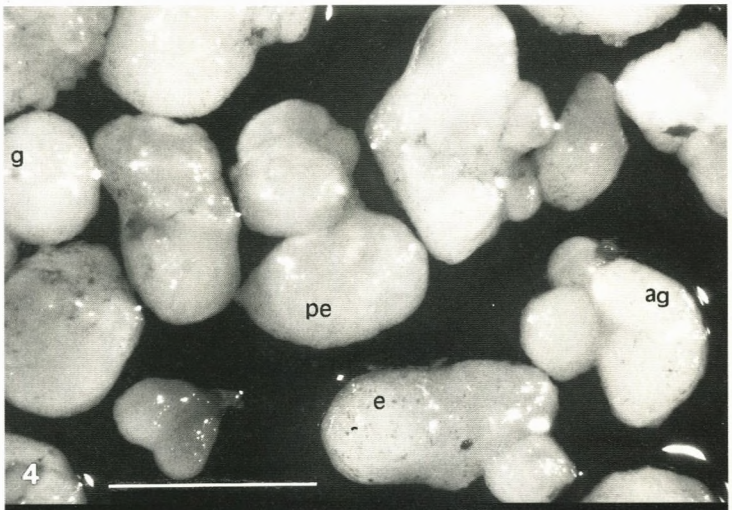

Figure 4.
Alstroemeria interspecific crossing between *A. presliana* and *A. ligtu*. Various stages of single embryos and small aggregates after 28 days in liquid BM3 medium. Globular embryo (g), elongated embryo (e), partly elongated embryo (pe), aggregate (ag). Bar = 5 mm.

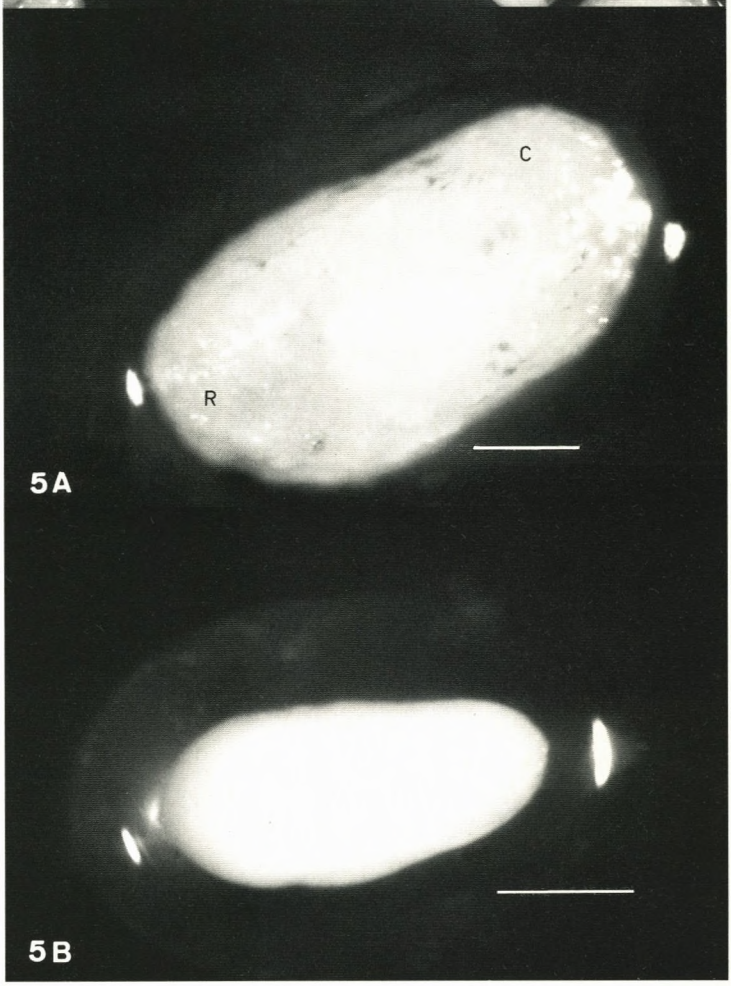

Figure 5. A-B
Alstroemeria. The elongated stage of an embryo developed in liquid culture (5A), resembles a mature zygotic embryo (5B). Cotyledon (c), root end (r). Bar = 1 mm.

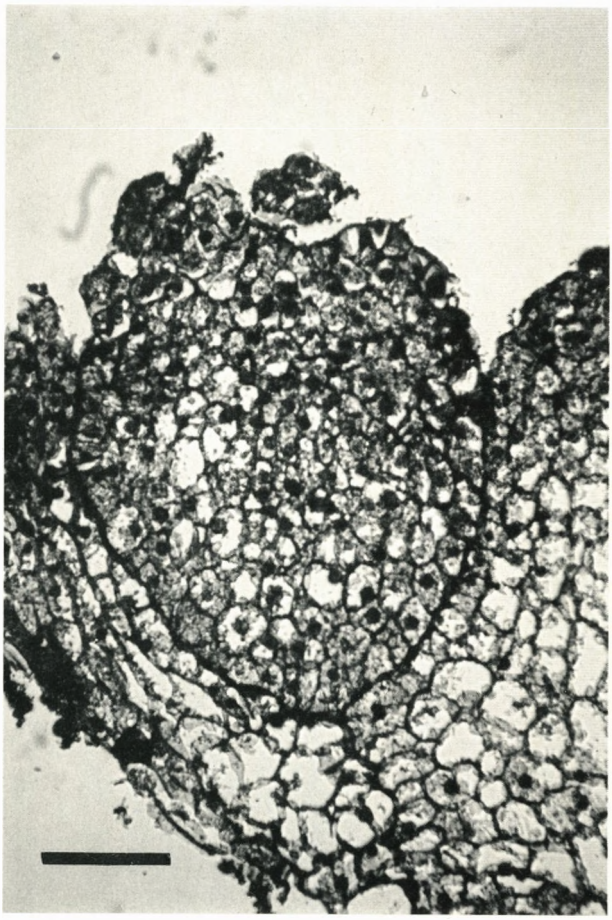

Figure 6. *Alstroemeria* interspecific crossing between *A. presliana* and *A. ligtu*. Globular (secondary) embryo embedded in the surface of an older embryo. Bar = 50 µm.

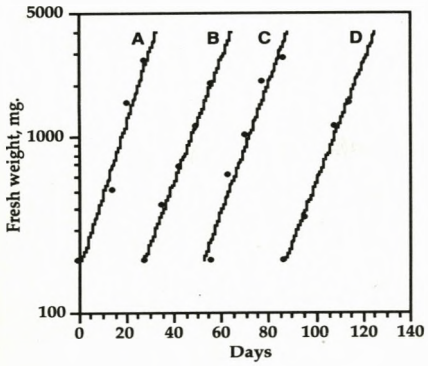

Figure 7. Kinetic of growth in liquid medium of *Alstroemeria* somatic embryos isolated from crossing A.
Each curve represents a set of 4 containers, initiated from one container belonging to the preceding set of containers. E. g. the 4 containers represented by curve C were all initiated from one of the 4 containers represented by curve B. Curve A represents only one container (the initial). New cultures were started each 28 days, in 20 ml BM3 medium. The subcultured tissue consisted of a mix of globular and elongated embryos and small aggregates. Medium was changed, and FW determined each 7 days.
Estimated fitted curves for the 4 growth curves:
Curve A: $y = 178.2 \exp(0.096 \text{ days})$; $R^2 = 0.97$
Curve B: $y = 218 \exp(0.080 \text{ days})$; $R^2 = 0.99$
Curve C: $y = 275 \exp(0.084 \text{ days})$; $R^2 = 0.93$
Curve D: $y = 198 \exp(0.078 \text{ days})$; $R^2 = 0.99$

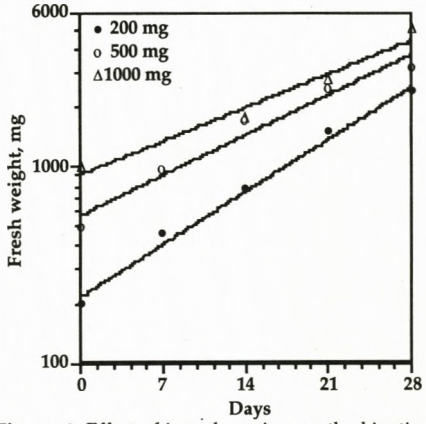

Figure 8. Effect of inoculum-size, on the kinetics of growth in liquid medium, of *Alstroemeria* somatic embryos isolated from crossing A. Fitted curves represent the data. Starting inoculum 200 mg (- • - • -) $y = 221.5 \exp(0.088 \text{ days})$; $R^2 = 0.99$; doubling time 8.1 days. Starting inoculum 500 mg (- o - o -) $y = 572.7 \exp(0.067 \text{ days})$; $R^2 = 0.97$; doubling time 10.7 days. Starting inoculum 1000 mg (- Δ - Δ -) $y = 921.3 \exp(0.056 \text{ days})$; $R^2 = 0.97$; doubling time 12.1 days. Number of containers varied: n = 34 for 200 mg; n = 4 for 500 and 1000 mg.

Both somatic embryos which had developed on abnormal plantlets and from "non germinating ovules" could be transferred to a semi solid BM3 medium where they grew from about 300 mg FW (globular stage) to 1400 ± 234 mg (n = 10, 5% level) after 1 month of culture. These calli, monthly subcultured on BM3, maintained approximately their 4x monthly multiplication rate and their regeneration ability during 18 months.

Growth of somatic embryos in liquid medium

Somatic embryos which had developed from abnormal germinating plantlets or which came directly from "non germinating ovules" were subcultured in liquid medium.

Initially, single globular embryos increased in size from 1 mm to 4-5 mm in diameter after 14 days of culture, and secondary embryos were visible on their surface after 21 days (Fig. 3). At the end of a 28 days culture cycle the initial globular embryos had developed to 4-6 mm aggregates of early stage embryos from which single globular or elongated embryos were released into the medium (Fig. 4). Elongated somatic embryos from the liquid cultures looked like mature zygotic embryos (Fig. 5 A and B).

Aggregates of embryos from liquid culture had a more smooth surface than their homologues on agar solidified medium, probably because of friction among the aggregates in the liquid.

Microscopical observations showed early-stage embryos embedded in the surface of the aggregates (Fig. 6). Elongated embryos were liberated into the liquid, leaving a characteristic little grove in the tissue.

Fresh weight increased exponentially with time during four runs of 28 days with medium exchange every 7 days (R^2 ranged from 0.93 to 0.99 for the four subcultures). The doubling time of the cultures was approximately 8 days (Fig. 7 and 8). The growth curves are without lag phase.

Preliminary results showed that the growth rate could be further increased by medium exchange every 4 days (results not shown).

The effect of inoculum-size was studied for an initial FW of 200 mg, 500 mg, and 1000 mg per 20 ml (Fig. 8). The growth rate decreased with increasing inoculum density. An inoculum density of 200 mg resulted in a doubling time of 8.1 days, whereas, with a inoculum of 1000 mg, the doubling time was 12.1 days (Fig. 8).

An inoculum density of 200 mg in 20 ml liquid medium (10 mg ml^{-1}) gave a final biomass of 117 ± 10 mg ml^{-1} FW (n = 28, 5%) after 1 month of culture. As the 200 mg inoculum were equivalent to about 40 embryos (macroscopically visible) the growth could also be expressed as an increase from 2 embryos ml^{-1} to 23 embryos ml^{-1} after 1 month.

The cultured tissues had a mean dry weight percentage of 10.2 ± 1.3% (n = 16, 5%). This high percentage of dry matter reflects the dominance of meristematic cells with dense cytoplasm and small vacuoles in the liquid cultures.

In some cultures, excretions coloured the medium ink dark a few days after medium change. The coloration was not due to contamination. Selection of white tissue (embryos) for initiation of new cultures could normalise coloration and growth. The growth curves presented in Fig. 7 and 8 have been obtained with "normalised" cultures.

Plant regeneration and acclimatisation

Ten percent of selected embryos (the elongated embryos in Fig. 4 and 5A) converted to plantlets with shoot and root after transfer to semi solid BM3 medium (Fig, 9 and 10). Selected embryos from liquid culture converted to plantlets with the same frequency as embryos from agar solidified medium. Germination capacity was maintained after 18 months of culture on agar solidified medium as well as after 9 months of liquid culture. Fifty plantlets in total of genotype A and B, and 10 of genotype C were successfully acclimatised and transferred to the greenhouse where they flowered normally without visible variation (Fig. 11).

Discussion

Lu & Bridgen (1996) maintained on auxin containing medium a loose type of *Alstroemeria* callus which was reported ideal for suspension culture, however, no further information related to the suspension culture was presented. In previous reports various combinations of growth regulators have been used in order to maintain long term cultures of somatic embryos (Hutchinson *et al.* 1994; Lu & Bridgen 1996; Schaik *et al.* 1996). As we have

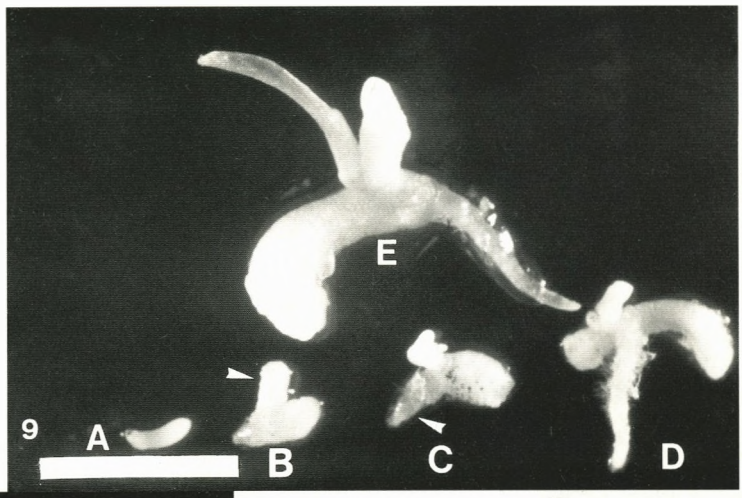

Figure 9. A-E "Germination" of somatic embryos of *Alstroemeria* interspecific crossing between *A. presliana* and *A. ligtu*. A: Elongated embryo; root end on the left. B: Development of the cotylodonary sheath (arrowhead). C: Emerging of primary root (arrowhead). D: Growth of primary root. E: Emergence of the first leaf. Bar = 5 mm.

Figure 10. Plantlet of *Alstroemeria* originating from a somatic embryo obtained from a crossing between *A. pulchella* and *A. pelegrina*. Note persisting primary root (arrowhead) and cotyledon. Bar = 5 mm.

Figure 11. Flowering plant originating from a somatic embryo obtained from a crossing between *A. presliana* and *A. ligtu*. Natural flowering (without flower induction by cold treatment) about 9 months after transplanting to greenhouse.

induced somatic embryos on media of various hormonal compositions especially in terms of auxin/cytokinin ratio and even without growth regulators (Table 1 and 2), it is possible that subtle variations in explant development were the determining factor for induction of somatic embryos, rather than the growth substances in the media.

Hutchinson *et al.*, (1994) found callus from their variety of *Alstroemeria* to be dependent on growth regulator supply for long-term maintenance of the embryogenic capacity. In our case, however, habituated embryogenic cell lines were obtained after successive selections on the growth regulator free medium. Habituated embryogenic cell lines have also been described for olive (Leva et al. 1995), Hemerocallis(Smith & Krikorian 1991), Citrus (Kochba et al. 1972; Kochba & Button 1974) and *Asparagus officinalis* (Delbreil *et al.* 1994). Habituated somatic embryogenesis in asparagus has been shown to be controlled by a mendelian dominant monogenic mutation that does not affect the phenotype at the whole plant level (Delbreil & Jullien 1994). Habituated cell cultures reduce the risk for somaclonal variation induced by the growth regulators in long term cultures. Mutations will then be restricted to the initial growth regulator treatment and thus do not deteriorate the regeneration potential.

A mutation in *Alstroemeria* giving improved ability to form somatic embryos, could be detected by using somatic embryo derived plants as donor plants for new somatic embryo induction as was done on *Medicago sativa* (Lupotto 1986) and in *Asparagus officinalis* (Delbreil & Jullien 1994).

Our original somatic embryos emerged from the white enlarged cotyledon of abnormal plantlets, but once isolated they grew by secondary embryogenesis with a stable multiplication rate. This multiplication rate was further improved by culture in liquid media.

We cannot really characterise our liquid cultures as suspension cultures which involves much smaller tissue agglomerates. It is, however, noteworthy that the kinetics of the liquid cultures were stable and exponential during four months of culture, and a low inoculum density gave shorter doubling time than high inoculum density. Usually in suspension culture increasing inoculum density, at least in a certain range, does not modify the doubling time. This is not true in our case, and the difference can probably be attributed to the fact that we did not establish real suspension cultures.

However, our cultures in liquid medium had a good growth. The final biomass reached after 1 month culture (130-221 g l^{-1} FW), was close to the theoretical maximum biomass value of 100-200 g l^{-1} FW for suspension cultures without medium change (Scragg 1995). Final biomass values may be increased by adding fresh medium to the culture at intervals (fed-batch culture); or the medium can be replaced without removing the tissue (perfusion culture) (Scragg 1995). Perfusion culture does actually describe our culture technique nicely, and the high biomass levels can probably be attributed to the regular medium change.

Our high biomass production is especially interesting, because it opens the possibility for further research in mass propagation of *Alstroemeria*, and for generating substantial quantities of highly susceptible target tissue for use in genetic transformation systems (Vasil 1995; Hunold *et al.* 1994). Early-stage somatic embryos of *Alstroemeria* were shown to be located on the sur-

face of primary embryos, making the cells from which the next generation of embryos develop accessible to transformation by *Agrobacterium*, as has been reported for asparagus (Delbreil *et al.* 1993), rice (Hiei *et al.* 1994), and maize (Ishida *et al.* 1996).

We conclude that culture of *Alstroemeria* somatic embryos in liquid medium has a growth potential which is highly interesting for both propagation and transformation.

Acknowledgement

The financial support from the Danish Directorate for Agricultural Develop-ment is gratefully acknowledged. The authors thank Dr. K. Kristiansen, Research Group Plant Breeding and Propagation, Årslev for manuscript comments.

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Rhizome formation from aerial shoots and somatic embryogenesis from rhizomes in *Alstroemeria x hybrida* L.

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Key words: Inca Lily; micropropagation; ornamental; rhizome; somatic embryogenesis.

Abstract

Micropropagation of *Alstroemeria x hybrida* is performed by rhizome division. However, the multiplication rate could be improved substantially if plants could be produced from other plant organs as well. We produced plantlets from apical meristems and nodes from *in vitro* grown aerial shoots. Plantlet formation occurred on several media compositions, however, addition of benzyladenine (BA) was imperative. More than 40% of the apical meristems could produce at least one plantlet after 58 days on media supplied with 10 or 20 µM BA together with 20 µM naphthaleneacetic acid (NAA). Wounded meristems produced a mean of 1.8 plantlets from 40% of the explants when the medium was supplied with 20 µM BA combined with 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D); and a mean of 1.3 plantlets from 100% of the explants when the medium was supplied with 50 µM BA combined with 20 µM 2,4-D.
Somatic embryos were induced from *in vitro* cultivated rhizomes of *Alstroemeria* on a basal medium with the growth regulator combinations: 10 µM 2,4-D + 50 µM BA; 20 µM 2,4-D + 20 or 50 µM BA. Only few embryos were found on each growth regulator combination. The somatic embryos germinated without secondary embryogenesis. Plants were obtained by both techniques and transplanted to the greenhouse.

Abbreviations: BA - 6-benzylaminopurine, IAA - indole-3-acetic acid; LSD - least significant difference, NAA - naphthaleneacetic acid, 2,4-D - 2,4-dichlorophenoxyacetic acid, SD - standard deviation.

Introduction

In *Alstroemeria*, the aerial shoots originate from the sympodial growing rhizome. Under natural growth conditions no above-ground plant parts are able to produce adventive shoots, so to propagate *Alstroemeria* it is necessary to divide the rhizome. Consequently, commercial propagation is mainly by micropropagation (for review see Monette 1992).

Alstroemeria micropropagation by rhizome division has been described (e.g.: Kristiansen et al. 1997; Pedersen et al. 1995; Monette 1992; Pierik et al. 1988; Gabryszewska & Hempel 1985). The multiplication rate in *Alstroemeria* is determined by the number of meristems on the rhizome when it is divided for a new subculture. Generally one *in vitro* rhizome can be divided in 2 to 3 every 4 weeks. The multiplication rate could be increased significantly if the aerial shoots also could be used for multiplication. An even higher multiplication rate could be achieved if cloning by somatic embryogenesis was possible. Immature and mature zygotic embryos have served as explants for induction of somatic embryos in *Alstroemeria* (Gonzalez-Benito & Alderson 1990, 1992 a, b; Hutchinson et al. 1994; Van Schaik et al. 1996), but the performance of plants from zygotic embryos is unknown at time of somatic embryo induction. Induction of somatic embryos from tissue of mature plants is necessary for cloning of true-to-type plants, but it has not yet been reported in *Alstroemeria*.

Here we report *in vitro* experiments with various parts of the plant as explants in order to find alternatives/supplements to the rhizome division.

Materials and methods

In vitro cultures of unnamed clones of Butterfly hybrids no 1 were maintained on a basal medium (Murashige & Skoog 1962) supplied with 2 g L^{-1} Gelrite® and 3% sucrose (Kristiansen 1995, Kristiansen et al. 1997) without growth regulators. At each subculture rhizomes were divided, so each new explant had one bud and 1-2 basal parts from aerial shoots which were excised prior to subcultivation. Cultures were maintained at least 4 months before any experiment. These stock cultures were grown in 150 ml plastic containers with 20 ml basal medium. Root meristems, root slices, nodes, internodes, leaves, and apical "meristems" were tested as explants from the *in vitro* grown plants; young inflorescences, petals and sepals from *in vivo* grown plants were tested as well. The apical "meristems" were taken from the apical part of the aerial shoots and consisted of the 3-4 mm stem which enclosed the meristem. For convenience this explant will be called "meristem" in the further text. The apical meristems were also cut longitudinally to obtain two wounded "half-meristems". Single buds from rhizomes were used in one experiment.

Results presented here include three experiments: A) Meristems and nodes were cultured on basal medium with all cytokinin/auxin combinations of 0, 5, 10, 20 µM BA; 10, 20 µM indole-3-acetic acid (IAA); 10, 20 µM NAA; 10, 20, 50 µM 2,4-D. B) Meristems were cultured on basal medium with all combinations of 10, 20, 50, 80 µM BA and 10, 20, 50 µM NAA. C) Wounded meristems and buds from rhizomes were cultured on basal medium with all combinations of 0, 10, 20, 50 µM BA and 0, 10, 20, 50 µM 2,4-D.

Five meristems or nodes were plated per Petri Dish with 20 ml medium. Five buds from rhizomes were plated in each 150 ml container with 20 ml medium. Experiments consisted of 1

or 2 replications. Treatments lasted for 6 or 8 weeks without subculture; then subculture was performed every 4 weeks on basal medium without growth regulators. Cultures were maintained at 20°C day and 15°C night; a 16-h photoperiod 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by a mixture of two types of fluorescent tubes: 60% Mazdafluor TFRS 40/Bi and 40% Philips TLM 33RS.

For the experiments A, B, and C observations were recorded respectively: 42; 58 and 97 days; 56 and 120 days after culture start. The percentage of explants producing roots, shoots, calli, embryos, and new plants with root, rhizome and shoot were recorded.

The two "half-meristems" which constituted one original meristem were observed as one explant, because unequal response could be attributed to unequal division of the original meristem. For rhizomes the recordings included a) "new meristems": the number of buds larger than 2 mm and b) "new explants": the potential number of new explants which could be taken from one original explant at time of observation (one new explant could include several meristems). Statistical analysis was performed using the JMP statistical package (SAS) for Macintosh.

Results

Almost any form of explant (root meristems, root slices, nodes, internodes, leaves, meristems, inflorescences, petals and sepals) were tested. However, only meristems of aerial shoots and the first distinguishable node below regenerated new plants (Ørnstrup *et al.* 1994). Thus, only results from meristems, nodes and rhizomes are further presented.

Meristems and nodes

New plants were produced from both nodes and meristems (Fig. 1 and 2). A clear effect of growth regulators was observed (Tab. 1).

Meristems produced roots without BA or at low BA levels (0-5 µM) and shoots at high amounts of BA (10-20 µM). Also it seems that the shoot regeneration is related to a certain amount of synthetic auxin as IAA induces fewer shoots than NAA and 2,4-D (Tab. 1).

In a subsequent experiment (B) BA and NAA were tested on apical shoot meristems. After 58 days 40-50% of these meristems had produced at least one new plantlet if they had been subjected to 10 or 20 µM BA. The level of NAA had no significant effect on explants subjected to 10 µM BA, however, 20 µM NAA gave significantly more plantlets than 10 or 50 µM NAA when combined with 20 µM BA (Fig. 3).

As the cultures grew older, the percentage of meristems producing new plants generally did not change substantially, however, the meristems which had been subjected to 50 µM NAA increased considerably their number of new plants attaining approximately the same level as the other NAA levels (Fig. 3).

Elongation was not scored during the experiments, but it was substantial. Meristems originally 3-4 mm long could produce a shoot of 60-80 mm during growth regulator treatment, with leaves reaching a length of 30-50

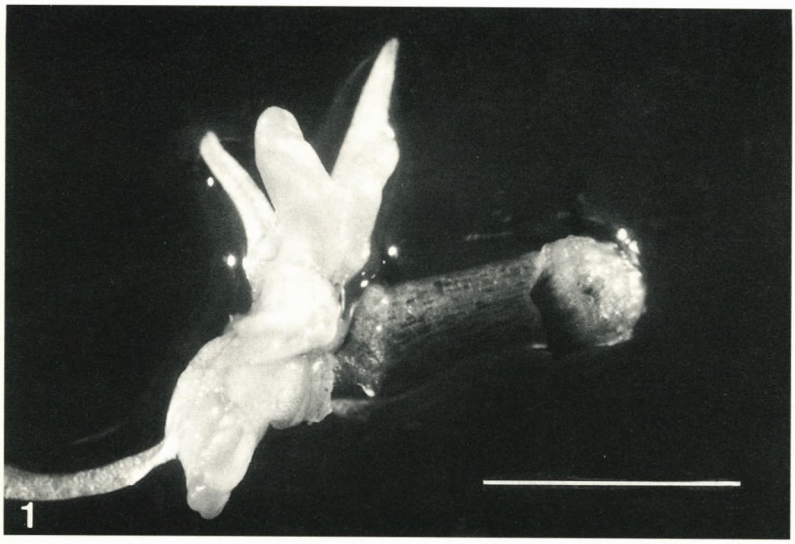

Fig. 1. Node from *Alstroemeria* with growing shoots from basal area after 42 days on medium with 10 µM BA and 20 µM NAA. New shoots come from the development and ramification of the preformed axillary meristems of the node. Roots will appear after further 14 days. Bar = 5 mm.

Fig. 2. Neoformed shoots and roots from meristem of *Alstroemeria* after 42 days on medium with 20 µM BA and 20 µM NAA. Arrow = root, arrowhead = shoot. Bar = 5 mm.

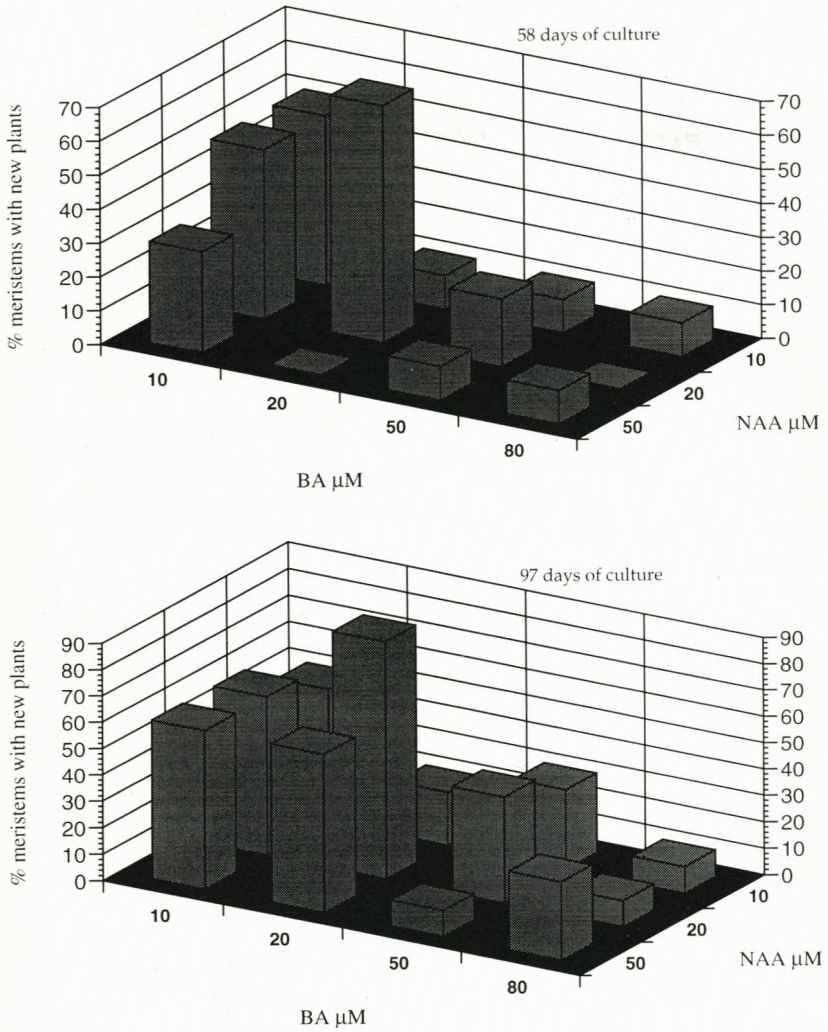

Fig. 3. Growth regulator effect on plant neoformation from apical shoot meristems after 58 and 97 days treatment. n = 10.

Tab. 1. The effect of various growth regulator combinations on root or shoot induction on apical meristem cultures. Scored after 6 weeks treatment. Total roots and total shoots represents respectively total number of roots and shoots from 5 explants.

BA µM	NAA µM	IAA µM	2.4-D µM	Total roots	Total shoots
0	0	0	10	17	0
0	0	0	20	0	0
0	0	0	50	0	0
0	0	10	0	0	0
0	0	20	0	0	0
0	10	0	0	2	0
0	20	0	0	4	0
5	0	0	10	0	0
5	0	0	20	0	1
5	0	0	50	2	0
5	0	10	0	0	0
5	0	20	0	0	1
5	10	0	0	0	0
5	20	0	0	0	0
10	0	0	10	0	2
10	0	0	20	0	0
10	0	0	50	0	4
10	0	10	0	0	0
10	0	20	0	0	1
10	10	0	0	0	4
10	20	0	0	0	6
20	0	0	10	0	5
20	0	0	20	0	4
20	0	0	50	0	4
20	0	10	0	0	1
20	0	20	0	0	0
20	10	0	0	0	0
20	20	0	0	0	2

mm. Shoots originated only from axils on the stems of the elongated meristems, however, roots could arise all over the stem.

Axillary buds. In some cases several new buds were produced already after 6 weeks giving something looking like a new rhizome (Tab. 1, 2 and 3). However, with time every developing axillary bud gave a new rhizome when transferred on the basal medium (Fig. 3).

Nodes were not affected by IAA (results not presented). However, nodes subjected to low level of NAA or 2,4-D (10 µM) produced roots at low level of BA (0-5-10 µM), but not at high level of BA. Increased level of 2,4-D apparently reduced the number of roots. Shoots were not formed on media lacking BA (Tab. 2). In the best hormonal condition, each node produced about one shoot which appeared to originate from the basal part of the stem (Fig. 2). Development was retarded by 2-3 weeks relative to meristems.

The wounded meristems ("half-meristems") were efficient in producing new plants, 7, 10 or 13 new plantlets from originally 10 meristems were found in several media (Tab. 3). Wounded meristems produced a mean of 1.8 plantlets from the responding 40% of the explants when the medium was supplied with 20 µM BA combined with 10 µM 2,4-D; and a mean of 1.3 plantlets from 100% of responding explants when the medium was supplied with 50 µM BA combined with 20 µM 2,4-D. New plants could not be induced without BA, but the response to the various BA levels was not significant ($p = 0.25$). However, the number of neoformed plants from the wounded meristems was influenced by the 2,4-D level ($p = 0.046$); and the high level (50 µM) of 2,4-D suppressed plantlet induction almost totally.

Tab. 2. The effect of various growth regulator combinations on root and shoot induction on nodes. Scored after 6 weeks treatment. n= 5. For legends see Tab. 1.

BA µM	NAA µM	2.4-D µM	Total roots	Total shoots
0	0	10	13	0
0	0	20	0	0
0	0	50	0	0
0	10	0	0	0
0	20	0	1	0
5	0	10	2	0
5	0	20	0	0
5	0	50	0	0
5	10	0	0	1
5	20	0	0	0
10	0	10	1	0
10	0	20	0	0
10	0	50	0	3
10	10	0	0	2
10	20	0	0	3
20	0	10	0	5
20	0	20	0	0
20	0	50	0	0
20	10	0	0	4
20	20	0	0	0

Tab. 3. New plants produced from wounded meristems subjected to various combinations of 2,4-D and BA. Scored after 120 days culture. The first 56 days with growth regulator treatment. n = 10.

2,4-D µM	BA µM	Responding explants %	Mean of new plants per responding explant	SD new plants
0	0	0	0	0
0	10	40	1.5	1.0
0	20	40	2.5	0.6
0	50	50	1.4	0.5
10	0	0	0	0
10	10	30	1.7	1.2
10	20	40	1.8	0.5
10	50	70	1.4	0.5
20	0	0	0	0
20	10	0	0	0
20	20	20	1.0	0.0
20	50	100	1.3	0.5
50	0	0	0	0
50	10	10	1.0	0
50	20	0	0	0
50	50	0	0	0

Rhizomes

The average number of new meristems and potential new explants induced on rhizome explants were significantly ($p < 0.0005$) higher on media without 2,4-D than on media supplied with 2,4-D, and this regardless of the BA level (Tab. 4). Cytokinin level showed no significant effect on either the number of new meristems ($p = 0.65$) or the number of new explants ($p = 0.79$) induced on rhizomes. The rhizomes could produce two varieties of calli a green and a yellow one (Tab. 5). The green callus was restricted to media with auxin. The yellow callus appeared rarely, but has in other cases given rise to somatic embryos (Ørnstrup & Jullien 1998). Rhizomes were the only explants able to produce somatic embryos. Embryos were found in three treatments: 10 µM 2,4-D + 50 µM BA; 20 µM 2,4-D + 20 or 50 µM BA (Tab. 5). These are very few embryo inductions. So nothing can be concluded in terms of growth regulator effect. But it is noteworthy that these media included both auxin and cytokinin. The induced embryos were isolated, but converted to plantlets before embryo cultures could be established. Plants established *in vitro* were transplanted to the greenhouse and yielded normal looking plants.

Tab. 4. Average number of new meristems and potential new explants induced on rhizomes with one bud. Scored after 120 days culture. The first 56 days with growth regulator treatment. n = 10.

2.4-D µM	BA µM	New meristems	SD new meristems	New explants	SD new explants
0	0	17.5	8.1	8.7	5.1
0	10	23.2	11.1	8.8	4.0
0	20	24.0	7.2	8.3	2.9
0	50	17.8	10.6	6.7	3.8
10	0	3.1	2.5	1.2	0.8
10	10	6.2	9.4	3.0	4.7
10	20	4.0	5.1	1.8	1.8
10	50	7.3	7.9	3.5	3.4
20	0	1.5	2.8	0.4	1.3
20	10	2.9	4.7	1.1	1.6
20	20	1.1	2.4	0.8	1.6
20	50	0.8	1.0	0.5	0.7
50	0	0.3	0.7	0.3	0.7
50	10	0.0	0.0	0.0	0.0
50	20	1.6	3.0	0.7	1.3
50	50	2.8	4.5	1.4	2.5

Tab. 5. Number of rhizome-explants with induced embryos, green callus or yellow callus. Scored after 120 days culture. The first 56 days with growth regulator treatment. n = 10.

2.4-D µM	BA µM	Embryos	Green callus	Yellow callus
0	0	0	0	1
0	10	0	0	0
0	20	0	0	0
0	50	0	0	0
10	0	0	5	0
10	10	0	3	0
10	20	0	2	0
10	50	1	8	0
20	0	0	4	1
20	10	0	4	0
20	20	1	6	1
20	50	1	2	1
50	0	0	10	0
50	10	0	7	0
50	20	0	7	1
50	50	0	0	1

Discussion

Induction of new plants from meristems and nodes of vegetative aerial shoots have not yet been reported in *Alstroemeria*. Ziv *et al.* (1973) used young inflorescence stems in their pioneer work, but this explant did not work in our hands. Vegetative tissue is a more reliable explant, available all year round *in vivo*, and as a surplus from *in vitro* rhizome multiplication as well. Although no optimal growth regulator combination could be found, a certain level (at least 10 µM) of BA seems essential for plant neoformation. The necessity for auxin was indicated by the fact that IAA induced fewer shoots than NAA and 2,4-D. In conclusion, the most efficient medium for multiplication of *Alstroemeria* by apical meristems has to include both cytokinin and auxin,

and it is probably their relative proportion which is important.
Reactivation of inactive axillary meristems can be done by disturbing the dominance imposed by the apical meristem. This can be done by deleting or wounding the apical meristem. Explants from wounded meristems ("half-meristems") produced around 2 new plants per explant and may be a good alternative to meristems or nodes. However, further research is needed to establish the best combination of growth regulators and explants.

Rhizomes were the only explants able to produce somatic embryos. The results were too sporadic to indicate the best growth regulator combination, but are very encouraging for further work. A hard yellow callus is often the last step before attaining somatic embryos (Remotti & Löffer 1995; Taylor *et al.* 1992) and may be considered as deformed primary somatic embryos. So, the appearance of a yellow callus may be an indication of media composition permitting somatic embryo induction. Also the incident of yellow callus and embryo induction on 20 µM 2,4-D should be noted. Auxin, and especially 2,4-D is commonly used for callus induction, so the single observation of the yellow callus on media without any growth regulator was surprising. Winski & Bridgen (1988) induced somatic embryos on media without growth regulators, however, they used immature zygotic embryos as explants. But, due to their unknown genotype, zygotic embryos are not useful for large scale multiplication. More genetic well-defined explants have to be used. Central slices of *Gladiolus* cormels were more effective than apical or basal slices as explants for induction of somatic embryos in *Gladiolus* (Remotti & Löffler 1995). The absence of meristems on the central slices was described as the reason. Slices from *Alstroemeria* rhizomes without visible meristems may be a possible explant for induction of somatic embryos. Callus has in *Alstroemeria* been reported from slices of flowering stems and mature ovaries (Ziv *et al.* 1973), from immature zygotic embryos (Winski & Bridgen 1988; Van Schaik *et al.* 1996) and from mature zygotic embryos (Gonzalez-Benito & Alderson 1990, 1992 a, b, Hutchinson *et al.* 1994). However, this is the first report of somatic embryos initiated from vegetative tissue of *Alstroemeria*.

Acknowledgement

The financial support from the Danish Directorate for Agricultural Develop-ment is gratefully acknowledged. The authors thank Dr. K. Kristiansen, Research Group Plant Breeding and Propagation, Årslev for manuscript comments.

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Summary and concluding remarks

The possibilities for *in vitro* cloning of asparagus (*Asparagus officinalis)* and *Alstroemeria x hybrida* are presented with special emphasis on somatic embryogenesis.

Induction of somatic embryos in asparagus was done from *in vitro* cultivated axillary meristems, which developed clusters of roots after a short NAA treatment. From the surface of these roots originated somatic embryos. Isolated embryos developed lines of somatic embryos, which were cultivated without growth regulators for more than two years.

In *Alstroemeria* somatic embryos were induced from immature zygotic embryos, although no specific treatment for the induction was found. The first induced somatic embryos produced cultures of embryos by secondary somatic embryogenesis. These cultures could be maintained on a basal medium without growth regulators.

Somatic embryos from *Alstroemeria* were also cultivated in liquid medium without growth regulators. One month culture in liquid medium resulted in a 12-fold increase in fresh weight.

Fu99ced from *in vitro* cultivated rhizomes of *Alstroemeria* on a basal medium with the following growth regulator combinations: 10 µM 2, 4-D + 50 µM BA; 20 µM 2, 4-D + 20 or 50 µM BA. From these rhizomes were only produced a few embryos, which germinated without secondary embryogenesis.

In both cases induction of the first somatic embryos needed a growth regulator treatment (which can be interpreted as a stress), but further culture of the embryos, from both asparagus and *Alstroemeria*, was possible without growth regulators. So, did the stress activate or suppress the same genes in these two plants? As mentioned in the introduction to somatic embryogenesis (Chapter 1), it is possible to induce somatic embryos by several treatments, even to the same species. So, the next question is: how can different treatments influence the same fundamental process (induction of somatic embryogenesis) in a plant? And further: how can one product (e.g. 2, 4-D) induce somatic embryogenesis in different species?

These questions have not yet been solved, however, a unifying theory was presented by Okkels (1988). According to Okkels (1988) the auxin treatment results in a successive demethylation of the DNA in the dividing cells -- and the result is callus. At a certain level of demethylation arises a special callus type, normally referred to as embryogenic. When the embryogenic callus is transferred to a medium without auxin, methylation is again possible and embryo development can initiate. The main problem in this hypothesis is that many different treatments of the initial explant can give rise to a embryogenic callus. The reader should consult Chapter 1 and the literature for further discussion of theories explaining the somatic embryogenesis.

Conversion of the somatic embryos to plantlets

A general difficulty in the work with somatic embryos is a low conversion frequency.
In asparagus neither abscisic acid treatment nor desiccation improved the conversion frequency. Norflurazon stopped chlorophyll synthesis and reduced growth of the somatic embryos, however, conversion frequency was not effected. The inhibition of the carotenoid synthesis reduces the ABA level; because the ABA synthesis needs carotenoids. As ABA plays an essential role in managing the fine equilibrium within the cell, especially during osmotic stress and embryo development, another way to influence the ABA level may be by desiccation or some sort of osmotic stress. We found that improved air exchange and culture on filter paper could improve the fresh weight of mature somatic embryos as compared to embryos cultured directly on the medium without air exchange. Further experimentation involving ABA and various forms of desiccation may in the future show the way to increased conversion rates in somatic embryos of both asparagus and other plants.

When the plantlets are big enough they have to be transplanted to the greenhouse or the field. This step represents a huge stress for the plantlet and precautions must be taken in order to insure a high survival rate. It is especially important to keep a high relative humidity in the acclimatisation area.

Plants regenerated from somatic embryos of asparagus have been transplanted to the field and will be evaluated the following years. It will be very interesting to compare the development and yield from plants propagated by seed, cloned by node culture and cloned by somatic embryogenesis.
As *Alstroemeria* is an ornamental plant, the most important feature for a regenerated plant is the general habitus and especially the flower. For pot plant production an even and fast flowering is very important. Because the somatic embryos arose from ovules we have not been able to evaluate the similarity of regenerated plants and their ancestors. However, a few (50) plants regenerated from somatic embryos of *Alstroemeria* interspecific crosses grew and flowered normally in the greenhouse.

Multiplication of *Alstroemeria* from shoots

When we tested the various possible explants for induction of somatic embryos an unexpected extra result was our discovery of the ability of apical meristems and nodes, from *in vitro* grown aerial shoots, to produce normal plantlets. Wounded meristems produced between 1.3 - 1.8 plantlets from 40 - 100% of the explants after 58 days.
The use of apical meristems from aerial shoots of *in vitro* grown rhizome cultures of *Alstroemeria* can improve their *in vitro* multiplication rate substantially in the future.

The final words

Asparagus and *Alstroemeria* have many similarities -- but also many differences.

Asparagus is a well studied plant, both in the field and *in vitro*, and almost all possible biotechnolical techniques have been applied to asparagus (see Chapter 2). This is not the case for *Alstroemeria*, and we can just hope that the work presented here can accelerate further research in *Alstroemeria*.

The transformation work on asparagus have shown great progress, and introduction of quality improving genes (e.g. ethylene insensibility) is now technically possible (Ayub *et al.* 1996).

If (cloned) high yielding plants also are able to produce a high-quality product, which can keep this quality a few days more than now, then, both the producer and the consumer will be happy.

We regret that the seed industry and pot plant producers have not found our results sufficiently interesting to finance further work on these plants.

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ISSN 1397-9876

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