INDIRECT SHOOT ORGANOGENESIS AND SELECTION OF SOMACLONAL VARIATION IN *Dieffenbachia*

By

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To my parents
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<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>2iP</td>
<td>N⁶-(Δ² – isopentenyl) adenine</td>
</tr>
<tr>
<td>BA</td>
<td>6-benzyladenine</td>
</tr>
<tr>
<td>BM</td>
<td>basal medium</td>
</tr>
<tr>
<td>CPPU</td>
<td>[N-(2-chloro-4-pyridyl)-N-phenylurea</td>
</tr>
<tr>
<td>GA₃</td>
<td>gibberellic acid</td>
</tr>
<tr>
<td>NAA</td>
<td>1- naphthalene acetic acid</td>
</tr>
<tr>
<td>PGR</td>
<td>plant growth regulator</td>
</tr>
<tr>
<td>TDZ</td>
<td>thidiazuron</td>
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A series of experiments were conducted to investigate the feasibility of selecting of somaclonal variants for new cultivar development in *Dieffenbachia*. In the first set of experiments, a protocol for indirect shoot organogenesis was established for *Dieffenbachia* cv. Camouflage. Maximum 96% callus formation frequency was observed on a basal medium supplemented with 5 µM TDZ and 1 µM 2,4-D. The maximum shoots regenerated per callus (7.9) was obtained on a basal medium supplemented with 40 µM 2ip and 2 µM IAA. In the second set of experiments, 4 *Dieffenbachia* cultivars were examined for the capacity for indirect shoot organogenesis and effects of genotypes, explant sources and plant growth regulators were investigated. There were significant genotypic effects on both callus formation and shoot regeneration. Cultivar Camouflage exhibited the greatest ability for indirect shoot organogenesis, while cv. Octopus had no capacity for shoot regeneration from calli. Only leaf explants taken from *in vitro* shoot cultures were capable of callus formation. Root explants failed to undergo indirect shoot organogenesis, regardless of cultivar. In the third set of experiments, somaclonal variation at the phenotypic level among *Dieffenbachia* plants regenerated via indirect shoot organogenesis was evaluated. Three types of somaclonal variations with different leaf variegation and color were observed in cv. Camouflage with a total somaclonal variation rate of
40.4%. Somaclonal variation in leaf shape was observed in cv. Camille with a somaclonal variation rate of 2.6%. No somaclonal variation was observed in regenerated plants of cv. Star Bright. The duration of callus culture of cv. Camouflage had no effect on somaclonal variation as variation rates between plants regenerated from 8 months and 16 months callus culture were similar. Our results indicated that selection of somaclonal variation has great potential for new cultivar development in *Dieffenbachia*. 
CHAPTER 1
LITERATURE REVIEW

Introduction

The genus *Dieffenbachia* belongs to the family Araceae and is one of the most popular ornamental foliage plants. Its popularity is largely attributed to its attractive foliar variegation, which is generally manipulated through breeding. Traditional breeding in *Dieffenbachia*, however, is hindered by its naturally-occurring dichogamy and long breeding cycle (Henny, 1988). High demand for new cultivars with novel foliar variegation has created a need for developing more efficient breeding methods. Selection of somaclonal variants from *in vitro* generated population could be an alternative means for new cultivar development. This study aimed at establishing regeneration methods for *Dieffenbachia* through indirect shoot organogenesis and examining the feasibility of selection of somaclonal variants from the regenerated population for new cultivar development. It is hypothesized that plants regenerated through indirect shoot organogenesis were associated with high somaclonal variation because of intermediary callus formation. Since indirect shoot organogenesis has not been established in *Dieffenbachia*, the first experiment was to establish a protocol for indirect shoot organogenesis in *Dieffenbachia*. The second experiment was to determine factors affecting indirect shoot organogenesis. The third experiment was to assess somaclonal variation among regenerated plants for potential new cultivar development.

*Dieffenbachia* Genus

Botany

*Dieffenbachia* genus, a monocot and a member of the family Araceae, is native to tropical regions of Central and South America (Chen et al., 2003b). It is a herbaceous perennial evergreen with thick stems bearing alternate leaves (Black, 2002). Flowers are unisexual and
consist of spadix and spathe. The spadix is a central fleshy spike, covered with many small staminate and pistillate flowers. The spathe is a modified bract and envelops the spadix until anthesis (Henny, 1988). Self-pollination is prevented by the naturally occurring dichogamy in this genus as female flowers mature earlier than male flowers. The leaves are broad and variegated with white marking or different patterns which are sheathed petioles and have a very striking appearance (Henny and Chen, 2003). Thus, the value of *Dieffenbachia* lies in its attractive foliar variegation. Additionally, *Dieffenbachia* requires low light levels for its growth. As a result, *Dieffenbachia* is widely used as living specimens for interior decoration and interiorscaping. *Dieffenbachia* is among the most popular ornamental foliage plants in the United States, continually ranking in the top five for annual wholesale value (Chen et al., 2002).

**Propagation**

*Dieffenbachia* can be propagated by both sexual and asexual methods. Seed propagation is not usually used in the production because of limited seed production and poor seed germination. *Dieffenbachia* can be easily propagated by asexual methods, by tip, cane cuttings and divisions, but such methods only produce a few cuttings or plants from each stock plant and require significant labor input. Asexual propagation can also carry over pathogens. The advantage of propagation by cuttings or divisions is that plants propagated are true-to-type.

Plant tissue culture technique can potentially overcome all these limitations of traditional approaches to *Dieffenbachia*. *In vitro* culture is becoming an important method for propagation of *Dieffenbachia* since 1980s. An initial application of tissue culture in propagation of *Dieffenbachia* was to eliminate systemic viral and bacterial pathogen. *Dieffenbachia cv.* Perfection derived from lateral bud and shoot tip culture were indexed 3 separate times on each of 4 different media, and plantlets lines free of tested fungi and bacteria were released. This line showed vigorous growth and branched more freely due to the depletion of fungi and bacteria.
(Knauss, 1976; Chase et al., 1981). Later, the tissue culture was mainly used to produce a large number of uniform and true-to-type healthy plants for commercial production on a year-round basis. Through modification of media and culture conditions, the propagation rate of Dieffenbachia had been greatly increased (Voyviatzi and Voyiatzis, 1989). Tissue culture also reduces greenhouse space needed for stock plant production and produces salable plants in a greater range of pot sizes.

In addition to providing true-to-type liners, tissue culture also produces off type plants. In the early application of tissue culture for propagation of Dieffenbachia, all off-type plants were rouged out to maintain the genetic fidelity of plants produced. It was later realized that these off-type plants could be a source for selection of somaclonal variation for new cultivar development. Since Larkin and Scowcroft (1981) advocated that somaclonal variation could be used as a promising tool for breeding to produce novel genetic variation, some somaclones with desirable features have been obtained by this method (Chen and Henny, 2006). It generally requires about 2 to 3 years for a new cultivar development compared to 7 to 10 years through traditional breeding (Henny et al., 2000).

**Breeding**

Progress in breeding of Dieffenbachia has been slow due to the long breeding cycles. The first hybrid Dieffenbachia Bausei was released in 1870 (Birdsey, 1951), since then only about 100 new cultivars have been developed. Hybridization is the most common method in producing new cultivars in Dieffenbachia, but this requires many crosses and careful selection. Naturally occurring dichogamy in this genus also makes this process very laborious and time consuming. It usually takes about 7-10 years for a new cultivar to be released (Henny et al., 1986; Henny et al., 1987a, b; Henny et al., 1988a, b; Henny et al., 1991a, b, c; Henny, 1995a, b). In addition to hybridization, new cultivars are also developed from sports or from somaclonal variation. For
example, cv. Camouflage is a mutant of Panther, and cv. Camille is a sport of cv. Marianne. Selection of somaclonal variation from \textit{in vitro} cultures has become an important method for new cultivar development. So far 20 cultivars of \textit{Dieffenbachia} derived from selection of somaclonal variants have been released (Chen and Henny, 2006). As the value of \textit{Dieffenbachia} lies in its aesthetic appearance, any changes in plant form, size, color or variegation pattern can be desirable traits for \textit{Dieffenbachia}.

\textbf{Developmental Pathways for Plant Regeneration}

There are generally 3 developmental pathways for \textit{in vitro} plant regeneration: 1) propagation from pre-existing meristems (shoot culture or nodal culture), 2) shoot organogenesis, and 3) somatic embryogenesis (non-zygotic embryogenesis). Proliferation from pre-existing meristems refers to production of shoots from shoot meristems (shoot tips) or axillary meristems (axillary buds) followed by rooting of individual shoot (Kane, 2000a). Shoot organogenesis is propagation from explants without pre-existing meristems through production and subsequent rooting of adventitious shoots (Schwarz and Beaty, 2000). Somatic embryogenesis regards to production of embryos that are not the result of gametic fusion (Gray, 2000). Any \textit{in vitro} regeneration system is associated with a certain degree of somaclonal variation and selection of somaclonal variants can be used as an important method for new cultivar development. The degree of somaclonal variation is highly related with the regeneration pathway. Plants regenerated from pre-existing meristems are usually true-to-type and this method is used for clonal micropropagation. Methods involving adventitious meristem formation or a callus phase result in plants with higher levels of somaclonal variation (Bouman and De Klerk, 1997).
Organogenesis

Definition

Shoot organogenesis refers to propagation from explants without pre-existing meristems through production and subsequent rooting of adventitious shoots. There are 2 types of shoot organogenesis, direct and indirect. Direct shoot organogenesis is the production of shoots from explants directly, while indirect refers to the formation of shoots indirectly from an intermediary callus that first develops on the explants (Kane et al., 1994).

Advantages of Shoot Organogenesis

Shoot organogenesis has the following advantages compared to traditional propagation and other in vitro propagation systems: 1) shoot organogenesis is more efficient than propagation from pre-existing meristems; 2) shoot production is more synchronous than somatic embryo formation; 3) indirect shoot organogenesis is associated with higher genetic variability and have more chance for selection of somaclonal variants; 4) survival rate is higher among plantlets produced via shoot organogenesis than those via somatic embryogenesis after transferred to greenhouse; 5) shoot organogenesis is more easily achieved in most species than somatic embryogenesis.

Phases of Shoot Organogenesis

A series of cellular events occur in the de novo shoot meristem formation process, and it can be demonstrated in the following model:

<table>
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<th>determination</th>
<th>shoots</th>
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<tr>
<td></td>
<td>dedifferentiation</td>
<td>induction</td>
<td>differentiation</td>
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There are generally 3 phases of shoot organogenesis, namely dedifferentiation, induction and differentiation. Dedifferentiation refers to differentiated cells in explants that dedifferentiate
and revert to a less committed, more flexible/plastic developmental state that may or may not involve callus formation. In the direct shoot organogenesis, some cells from explant tissues dedifferentiate directly to produce a new primordial, while in the indirect shoot organogenesis, cells from explants dedifferentiate to give rise to callus formation from which the new shoots are produced. As a result of dedifferentiation, some of these dedifferentiated cells in explants may become competent and capable of responding to specific inducing signals. Plant growth regulators are one of these inducing signals through which cells become competent. It has been noticed in the induction of adventitious shoots from immature leaves of cassava that no adventitious shoots could be obtained on regeneration media if leaf explants were cultured on induction media for 1-3 days, indicating that cells has not become competent in such short time. However, after culture for 4 or more days on induction media, explants showed regeneration (Ma and Xu, 2002).

The span between the time cells become competent and when they become fully determined for primordial production refers to as the induction phase. During the induction phase, a competent cell or a group of competent cells become committed to a unique developmental fate on the stimulus of inducing signal. At the end of induction phase, cells become fully determined and capable of shoot organogenesis even with the removal of inducing signals (Schwarz and Beaty, 2000).

During the differentiation phase, morphological differentiation and development of the nascent organ occur. In their study on morphogenesis in petiole derived callus of Amorphophallus rivieri Durieu, Hu et al (2005) observed that compact callus consisted of 3 components: epidermis, subepidermis and inner parenchyma cells; cells in subepidermis started to divide to gave rise to the formation of a long and narrow meristematic zone after 1 week of
culture on differentiation medium; the meristematic masses then became more organized and formed meristematic domes by continuous anticlinal and periclinal division; the periclinal cell divisions on the flanks of the meristems resulted in leaf primordial formation after culture for 4 weeks. Through scanning electron microscopy, a group of regenerating shoot buds with distinct epidermal layer, unicellular trichomes, and stomata has been observed in callus cultures of *Flacourtia jangomas* (Lour.) Raeusch. The presence of stomata on the epidermal layer is thought to be a striking feature for shoot formation (Chandra and Bhanja, 2002). In a study on organogenesis induced from mature zygotic embryo-derived leaflets of peanut, Chengalrayan et al. (2001) reported that during the initial 4 days of culture, cells of the explants enlarged; mesophyll cells underwent anticlinal division giving rise to globular cell cluster on day 7 of culture; subsequent periclinal and anticlinal divisions resulted in dome-shaped shoot meristems in 15 days; well-developed shoot buds formed after 21 days of culture.

**Factors Affecting Shoot Organogenesis**

There are many factors which affect explant tissues becoming competent, and eventually determined to complete shoot formation.

**Genotypes**

Genotype has the most influence on capacity for shoot organogenesis *in vitro*. Some genotypes are easily cultivated *via* shoot organogenesis while others are either recalcitrant or exhibit no capacity for shoot organogenesis (Thao et al., 2003; Landi and Mezzetti, 2005). Significant differences in growth rate, color and structure of calli were found among 4 carnation genotypes tested and these differences were also responsible for differentiation of calli into a certain organogenic pathway (caulogenesis, shoot organogenesis or rhizogenesis). Callogenesis was not only a result of dedifferentiation of explant tissues, but also an essential preparatory
stage for in vitro morphogenesis. Callus type can be a prediction for its organogenic potential (Kallak et al., 1997).

Among 38 carnation cultivars tested for shoot organogenesis from nodal explants, 81.6% of them showed a high regeneration ability (> 50% and 4 shoots per node segment), and 18.4% showed low shoot regeneration (< 50% and 4 shoot per node segment) (Nontaswatsri et al., 2002). Phillips (2004) concluded that specific genes were involved in dedifferentiation, acquisition of competence and induction stages of shoot organogenesis. Some genotypes may lack the genes required for shoot organogenesis or these genes may not be functional. Plants of different genotypes may possess different endogenous plant growth regulator levels and this might be responsible for differences in response in vitro.

**Explants**

The developmental stage and type of explants play a critical role in the success of shoot organogenesis. The capacity of cells to become competent for regeneration may be completely lost once they mature to a point due to the elimination of totipotency of cells. In general, explants taken from less differentiated, immature tissues are easily induced and regenerated via direct shoot organogenesis. Explants derived from highly differentiated tissues are less responsive in vitro, and usually undergo indirect shoot organogenesis. In study on the effect of explant types on shoot organogenesis of Vigna unguiculata, Choi et al (2003) reported that the highest frequency of shoot organogenesis was obtained from single whole cotyledon explants (67.5%); the proximal halves of cotyledon (50.1%) showed better regeneration than distal halves (30.1%); no shoots formed from one or two cotyledon halves with embryo axis or embryo axis alone. Explants taken from stock plants of different developmental stages possess different endogenous hormonal level which affects their response in vitro. Differences in the ability to produce adventitious shoots among different explant types were also noted in shoot organogenesis in
eggplants. On MS basal medium supplemented with 0.2 µM TDZ, the percentage of explants that developed buds and the number of buds per explant was 100% (75-100 buds per explant) for leaf, 100% (75-100 buds per explant) for cotyledon, 5% (1-25 buds per explant) for hypocotyl, 20% (25-50 buds per explant) for epicotyl, and 65% (1-25 buds per explant) for node explants respectively (Magioli et al., 1998).

**Plant Growth Regulators**

Plant growth regulators are the most important inducing signal for shoot organogenesis. Dedifferentiation, induction and development of shoots or roots are regulated by both endogenous and exogenous growth regulators. The type of plant growth regulators and their interaction play an important role in dedifferentiation, induction and development of shoots or roots (Khanam et al., 2000). It is generally believed that cytokinins are beneficial for shoot formation, while auxins stimulate callus formation, root formation and somatic embryogenesis. The ratio of cytokinins to auxins is also critical in determining shoot versus root formation. A high cytokinin to auxin ratio promotes shoot meristem formation, while callus or root meristems are formed when the cytokinin to auxin ratio is low. Adventitious shoots were induced on media containing only BA or BA as the major PGR, while somatic embryos were produced on 2,4-D containing media in morphogenesis from leaves of cassava (Ma and Xu, 2002). It has been reported that different combination of auxins (NAA, IAA, 2,4-D) and cytokinins (BA, kinetin) at 0, 1, 2 mg l⁻¹ levels resulted in either direct adventitious shoot formation, callus formation, or indirect adventitious shoot formation (Makunga et al., 2005). Both exogenous cytokinin and auxin were required for shoot organogenesis from petiole explants in *Nymphoides indica*. BA was most effective in stimulating adventitious shoot formation compared to 2iP and kinetin (Jenks et al., 2000). In their study on the effects of cytokinin/auxin combination on organogenesis in *Duboisia myoporoides*, Khanam et al (2000) reported that the auxins 2,4-D and
NAA were more effective than IAA or IBA for callus formation, the greatest number of shoots were induced with 10 µM BA and 1 µM NAA among the combination of BA (0, 0.1, 1, 10) and NAA (0, 1, 10) examined.

**Somatic Embryogenesis**

**Definition**

Somatic embryogenesis is the production of embryos not resulting from gametic fusion (Merkle et al., 1990). Asexual embryogenesis is known to occur naturally in many plant species and many different names are used by different authors to describe this phenomenon in different species, such as apomixes, polyembryony, adventive, sporophytic and nucellar embryony. Many plant tissues, such as the nucellus, inner integument, synergids, antipodals, endosperm, and suspensor have been observed to naturally give rise to asexual embryos (Tisserat et al., 1979). Asexual embryogenesis can also be obtained in vitro culture and this process is usually called somatic embryogenesis. Since the first report of somatic embryogenesis in carrot cell culture in 1958 (Steward et al., 1958a, b), in vitro somatic embryogenesis has been reported in over 100 species (Krishnaraj and Vasil, 1995; Merkle et al., 1995). There are 2 types of somatic embryogenesis, direct or indirect. Direct somatic embryogenesis refers to somatic embryos produced directly from cells of explant tissues, with no intervening callus. Indirect somatic embryogenesis is characterized by the production of callus from explant tissues first, from which somatic embryos form (Merkle, 1997). Somatic embryogenesis has been achieved in a number of economically and ornamentally important plant species, but studies on *Dieffenbachia* has not been reported.

**Stages of Somatic Embryogenesis**

There are generally 3 developmental stages in somatic embryogenesis in vitro: induction, development and maturation (Zimmerman, 1993). Explants are composed of heterogeneous cell
types. During the induction stage, undifferentiated cells from explants can be induced to become embryogenically determined and somatic embryos can be obtained directly from these cells via direct embryogenesis. Differentiated cells from explants undergo dedifferentiation, meristemation and redifferentiation into embryogenic cells. In some cases, callus is formed first from explant tissues, and somatic embryos subsequently form. This process is referred to indirect somatic embryogenesis (Kohlenbach, 1985; Merkle, 1997). Explants may also contain a portion of pre-embryogenic determined cells derived from ovular or embryo tissues that may develop into embryos under a suitable condition (Bhojwani and Razdan, 1996). Somatic embryogenic cells can be distinguished from non-embryogenic cells by the following features. Embryogenic cells usually contain dense cytoplasm, prominent nuclei, thickened cell wall and are less vacuolated (Mooney and Van Staden, 1987).

During the development stage, a single embryogenic cell undergoes a series of transverse and longitudinal divisions, passes through globular, heart, torpedo, and cotyledonary stages for dicots or globular, scutellar and coleoptilar stages for monocots, and finally forms a bipolar structure with root and shoot meristems on opposite end which has the capacity to reproduce entire plants (Arnold et al., 2002).

During the maturation stage, the somatic embryos accumulate reserve and achieve desiccation tolerance (Ammirato, 1974). This is very important for successful germination and subsequent growth. The maturation stage in somatic embryogenesis has just been realized recently as an important stage because the degree of maturation can significantly affect the germination capability of somatic embryos (Bhojwan and Razdan, 1996). Normal looking somatic embryos may be physiologically immature. A period of reversible arrested growth is
necessary for proper germination and regeneration. Without such developmental stages, somatic embryos usually germinate precociously and finally die (Gray and Purohit, 1991).

**Morphology and Physiology of Somatic Embryos**

Although somatic embryos share many histological and morphological characteristics of zygotic embryos, they differ in several aspects. Unlike a zygotic embryo which has a suspensor and nourished *via* a suspensor, a somatic embryo may not have a suspensor or has an abnormal suspensor. Somatic embryos are typically formed from a proembryonal complex. A somatic embryo may grow directly from a proembryonal complex without the formation of suspensor (Thomas et al., 1972). In other cases, somatic embryos may be supported above explants by either a narrowed, not easily observed suspensor, or a very broad suspensor (Hakman et al., 1985). Cotyledons may develop abnormally, including multiple cotyledons, fused cotyledons or unequally sized pairs (Rashid and Street, 1973).

Somatic embryos have a less compressed and narrowed appearance than zygotic embryos due to the lack of protective seed coats (Gray and Purohit, 1991). Somatic embryos tend to develop asynchronously. Several stages are present in cultures at any time thus somatic embryos do not mature altogether (Profumo et al., 1986). Additionally, somatic embryos usually germinate continuously after they are formed without a quiescent resting phase typically observed in zygotic embryos. This leads to precocious germination and plants usually die after a certain period of time (Gray, 1989). Gray et al (1993) also noticed that certain parts of embryos might precociously germinate before full embryo maturation, such as trichome development on hypocotyls and cotyledons, hypocotyl elongation, cotyledon elongation, premature root emergency in their study on somatic embryogenesis in *Cucumis melo*.
Histological Studies on Shoot Organogenesis/Somatic Embryogenesis

Plantlets regenerated in vitro from adventitious meristems may originate either from shoot organogenesis or somatic embryogenesis. In shoot organogenesis, shoots are usually formed first, then roots are induced from the base of shoots, with a well-developed vascular connection between shoots and maternal explants. In somatic embryogenesis, shoots and roots are derived from embryogenic shoots and roots on bipolar structure of somatic embryos. There is no vascular connection between somatic embryos and parental explants (Puigderrajols et al., 2000; Chengalrayan, 2001). Before concluding that regenerants from in vitro culture have a somatic embryogenic origin, it is necessary to provide convincing histological evidence to prove it. A visual similarity between the morphological appearance of the regenerating structures and somatic embryos is insufficient to draw the conclusion of somatic embryogenesis. In his studies on morph-histological study of somatic embryo-like structures in hypocotyl cultures of Pelargonium (ornamental plants), Haensch (2004a, b) found that although somatic embryo-like structures were produced from hypocotyls, histological examination confirmed the lack of bipolarity and revealed vascular connections to the explants and concluded that there was no proof of somatic embryogenesis.

Like zygotic embryogenesis, somatic embryogenesis is assumed to pass through the same characteristic stages, and the demonstration of these key stages can be used as the primary criteria for confirming somatic embryogenesis. The ontogeny of somatic embryogenesis can be revealed by scanning electron microscopy or light microscopy. The normal somatic embryo development from globular, to heart-, to torpedo-shaped and finally cotyledon stage embryo has been described through scanning electron microscope in safflower (C. tinctorius L.)(Mandal and Gupta, 2002). Histological events during somatic embryo development in Serbian spruce (Picea omorika) have been recorded. Each somatic embryo initials formed on the cotyledon surface
after an unequal transversal division of a cell. The smaller, apical cell gave rise to apical dome and later on filamentous suspensor (Budimir, 2003). Somatic embryos with plumule and root pole have been documented in *Musa* spp. after 50 days of culture by passing through globular stage (Lee et al., 1997). The attainment of a globular appearance is regarded as one of the first key features of somatic embryo development. The presence of protoderm, the outmost layer of a developing somatic embryo, is also considered as a unique feature of somatic embryo development, and it is believed that protoderm has a function of regulation of further embryo development by applying physical and cell division limitation (Sharma and Millam, 2004).

The developing somatic embryos had no detectable vascular connection with the mother explants (Quiroz-Figueroa et al., 2002). Two cell types can be observed under light microscope, embryogenic and non-embryogenic cells. Embryogenic cells are smaller, compact, and possess relatively dense cytoplasm, densely stained nuclei, and small invisible vacuoles. Non-embryogenic cells usually have large vacuoles and unclear cytoplasm. Embryogenic cells may form embryogenic masses (EMS) by continued cell division. These actively dividing cells may develop into globular masses, forming proembryoids, but only a very limited number of these embryogenic cells may give rise to meristem-like structure (meristemoid), and differentiate into different staged embryos (Onay, 2000).

**Somaclonal Variation**

Variation among plants regenerated from tissue culture has been called somaclonal variation (Larkin and Scowcroft, 1981). From its origin, it can be deduced that somaclonal variation occurs in the period before the formation of meristematic tissues and terminated with formation of meristematic tissues (Bouman and De Klerk, 1997). Somaclonal variation is a random phenomenon that can occur at any location in the genome (De Schepper et al., 2003). Assessment of somaclonal variation can be achieved by analysis of phenotype, chromosome
number and structure, proteins, or direct DNA evaluation of plants (Bouman and De Klerk, 1997). The extent of phenotypic variation is usually determined as the percentage of plants showing aberrations from the parental plant for one or more defined characteristics. Plants with the deviant phenotypes are known as somaclones or somaclonal variants.

**Origin of Somaclonal Variation**

**Cultivars**

The potential for somaclonal variation varies among cultivars (genotypes). In their study on chromosomal instability in regenerants from *Medicago media* Pers, Najaran and Walton (1987) found there was significant difference in the ability to produce somaclones among cultivars, being 9.4%, 10%, 20% and 64% for the 4 cultivars tested respectively.

**Explant Sources**

As mentioned previously, explants usually are composed of many cell types and these cells may vary in differentiation and even ploidy levels. Plants regenerated from a single explant may originate from different cells. Generally speaking, explants with pre-existing meristematic tissues such as axillary buds, shoot tips and meristems show less variation than explants that have no pre-existing meristematic tissues such as leaves, roots or stems (Skirvin et al., 1994). Piccioni et al (1997) reported that no somaclonal variants were observed in alfalfa plants regenerated from axillary branching propagation, but plants regenerated from callus derived from petioles showed greater variation (23%) analyzed by RAPD markers. In chrysanthemum (*Chrysanthemum morifolium*), plants regenerated from pedicel explants showed little variation from control plants, but considerable variants were found among plants regenerated from petals. These variants flowered earlier and produced more flowers than control plants (De Jong and Custers, 1986). Tubers produced on potato plants regenerated from leaf, stem, rachis and tuber pieces had different skin color. Regenerants derived from stem, leaf, tuber piece produced tubers with pink-
red color as control plants while regenerants from rachis produced white, red-splashed white and mixed colored tubers (Wheeler et al., 1985).

Callus

Somaclonal variation is quite often associated with indirect in vitro regeneration involving an interviewing callus stage. During this period, differentiated cells undergo dedifferentiation, induction, redifferentiation (Rout, 1999). Bouman and De Klerk (2001) showed that the rate of somaclonal variation among Begonia regenerated via somatic embryogenesis was 1.5% but 10.6% for regenerants derived from the callus stage.

Duration of the Tissue Culture Phase

There is a general trend that somaclonal variation increases with the length of time that a culture has been maintained in vitro. In a study on the genetic instability during embryogenic cloning of celery, Orton (1985) noticed that if calli, derived from immature petiole segment, were maintained for 6 months by a series of transfer, 84% of the callus cells were karologically indistinguishable from the control, the remaining 16% exhibited chromosome loss or fusion, only 1 regenerated plant out of 95 showed an abnormal phenotype. After 12 months in culture, 97% callus cells were karologically distinguishable from the control, most were aneuploidy and all callus cells lost the capacity to produce embroids.

Measurement of Somaclonal Variation

Phenotypic Variations

Since Skirvin and Janick (1976) advocated that somaclonal variation could be used as a promising tool for breeding, some somaclones with desirable features have been obtained. The variation could be change in foliar variegation pattern, modification of flower color and size, alteration of leaf shape, increase in lateral shoots, and change in overall plant form (Griesbach et al., 1988; Khalid et al., 1989). New somaclonal variants with desirable dwarf and bushy
phenotype of lisianthus (*Eustoma grandiflorum*) have been obtained from leaf, stem culture. Naturally, all cultivars of lisianthus have a long stem with only apical branches. Application of growth retardants and pinching are required to stimulate basal branch formation for its ornamental values (Griesbach and Semeniuk, 1987). Khalid et al (1989) produced new somaclonal variants of chrysanthemum with novel lilac-colored ray florets using petals as explants. Dwarf daylily (*Hemerocallis*) has been selected from callus derived from shoot tip culture (Griesbach, 1989). Arene et al (1993) obtained rose variants with altered petal number, color and having dwarf growth habit from callus of *Rose hybrida* cv. Meirutral.

Tremblay et al (1999) reported that several morphological changes including dwarfism, fascination, variegate patterns, height alternation, modified branch angle, and bushy shape were observed in somatic embryogenesis of spruce species. Griga (2000) noted that morphological alternations including altered leaflet shape, changed stipule morphology, shortened internode, irregular leaf position on the stem, shortened flower stalk were found in pea (*Pisum sativum*) plants regenerated from immature zygotic embryo culture. Somaclonal variants with increased disease resistance and higher yield traits have been obtained from wheat somatic embryogenesis (Arun et al., 2003).

**Changes at Chromosomal Levels**

Somaclonal variation can be assayed at the chromosomal level. Some somaclonal variations result from changes in the chromosome number. Polyploidy and aneuploidy are quite often associated with morphological modification. Moyne et al (1993) reported that tetraploid, aneuploid and polyploidy cells were found in cultures of rose. Diploidization, aneuploidization and polyploidization have been observed in cultures of *Larix decidua* initiated from megagametophyte explants (Von Aderkas and Anderson, 1993). Except changes in chromosome number, alternation in chromosome structure, such as point mutation, chromosome breakdown
and reunion, deletion, and insertion have been observed in plants derived from somaclonal variation (Bouman and De Klerk, 1997).

Variations at DNA Levels

Changing DNA sequence is a fundamental event responsible for much of the reported somaclonal variation among regenerated plants. DNA sequence changes or variations include mutations involving one or a few nucleotides, deletions and insertions caused by unequal crossover, or activity of transposable elements. The precise way to determine mutations at DNA levels is to sequence the gene of interest. An alternative approach is to detect variation at DNA levels by electrophoresis of proteins and/or by PCR amplification of nucleic acid segments. The protein electrophoresis implies the existence of mutant alleles by virtue of changes in gene products. Isozyme analysis is a frequently used method of studying certain gene product changes during in vitro culture (Mangolin et al., 1997; Chen et al., 1998; Azeqour et al., 2002; Feuser et al., 2003). A series of PCR-based techniques have been developed over the years for genotyping and fingerprinting purposes. Among the PCR-based methods, RAPD and AFLP have been used to analyze genetic variation among regenerants of a wide range of plant species (Chen et al., 2006). Somaclone specific fragments can actually be cloned, and nucleotide sequences can be analyzed to determine exactly what changes occur at DNA sequence level. Fragment cloning was performed in variants regenerated from maize, pea, and tomato (Gostimsky et al., 2005).
CHAPTER 2
PROTOCOL ESTABLISHMENT FOR INDIRECT SHOOT ORGANOGENSESIS FROM LEAVES OF Dieffenbachia CV. CAMOUFLAGE

Introduction

The genus *Dieffenbachia*, a member of the family Araceae, is composed of 30 species native to South and Central America (Mayo et al., 1997). *Dieffenbachia* has been produced as an ornamental foliage plant for interiorscaping since 1864 (Birdsey, 1951) and consistently ranks among the top five most popular foliage plant genera based on annual wholesale value (McConnell et al., 1989; USDA, 1998). In addition to its tolerance to low light levels, another factor contributing to its popularity is the increasing release of new and attractive cultivars that provide consumers with a wide range of selection for novelty.

Interspecific hybridization has been the primary method of developing new cultivars (Henny and Chen, 2003). Hybridization of *Dieffenbachia*, however, has been hampered by naturally occurring dichogamy, long breeding cycle, and limited seed production (Henny, 1988). With the commercial application of in vitro propagation of *Dieffenbachia*, new cultivars have been released following selection of somaclonal variants (Chen and Henny, 2006). The frequency of somaclonal variants is generally high, and the time required for a new cultivar release can be only 2 to 3 years compared to 7 to 10 years required using traditional breeding methods (Skirvin et al., 1994; Chen et al., 2003a). Chen et al (2004) analyzed genetic relatedness of some cultivated *Dieffenbachia* using amplified fragment length polymorphism (AFLP) and found that cultivars selected from somaclonal variants differ genetically from their parents.

Successful use of in vitro techniques for producing somaclonal variants depends on the establishment of an efficient method for regenerating a large number of plants indirectly from an intervening callus stage (Maralappanavar et al., 2000; Niwa et al., 2002; Hossain et al., 2003; Anu et al., 2004; Arce-Montoya and Rodriguez-Alvarez, 2006; Hammerschlag et al., 2006).
Although plant regeneration via indirect shoot organogenesis has been achieved in a vast array of plant species, a protocol for indirect shoot organogenesis has not been developed in *Dieffenbachia*. Currently commercial *in vitro* propagation of *Dieffenbachia* is through shoot culture (Knauss, 1976; Chase et al., 1981; Voyiatzi and Voyiatzis, 1989; Henny et al., 2000). The objective of this study was to establish a protocol for inducing indirect shoot organogenesis in *Dieffenbachia* cv. Camouflage.

**Materials and Methods**

**Media and Sterilization Conditions**

A basal medium (BM) consisting of MS (Murashige and Skoog, 1962) mineral salts, 0.4 mg l\(^{-1}\) thiamine, 2.0 mg l\(^{-1}\) glycine, 100.0 mg l\(^{-1}\) myo-inositol, 0.5 mg l\(^{-1}\) pyridoxine, 0.5 mg l\(^{-1}\) nicotinic acid, and 25 g l\(^{-1}\) sucrose in combination with plant growth regulators was used. Medium was adjusted to pH 5.8 with 0.1 N KOH prior to the addition of 6 g l\(^{-1}\) TC agar (PhytoTechnology Laboratories, Shawnee Mission, KS) and autoclaved at 1.2 kg cm\(^{-2}\) for 20 min.

**Plant Materials and Establishment of Shoot Cultures**

Stem segments about 10 mm long, containing lateral buds, were cut from stock plants of *Dieffenbachia* cv. Camouflage. The stock plants were maintained in a shaded greenhouse with a maximum irradiance of 345 µmol m\(^{-2}\) s\(^{-1}\) under natural photoperiod (10 to 14.5 h light) and a temperature range of 20 to 31°C. Lateral buds were excised, rinsed under running water for about 10 min and used as explants to initiate *in vitro* shoot cultures. After surface sterilization in aqueous 1.2% sodium hypochlorite (20% v/v Clorox Ultra) containing several drops of Tween 20 for 10 min on a shaker and rinsing 3 times, 5 min each, with sterile water, lateral buds were further trimmed by removing the outermost one or two bud scales. Lateral buds still attached to a 2 mm\(^2\) thick square of stem tissue were cut and placed individually into 25 × 150 mm culture...
tubes containing 15 ml BM supplemented with 80 µM 2iP and 2 µM IAA. Cultures were maintained under a 16 h light photoperiod at 40 µmol m⁻² s⁻¹ provided by cool white fluorescent lamps (General Electric F20WT12CW) at 22 ± 3°C. Shoot clusters were divided and transferred to the same fresh medium every 8 weeks to increase in vitro stock shoot cultures.

**Indexing of Established Cultures**

Following the establishment of in vitro shoot cultures, cultures with visually detectable contamination were immediately discarded. Cultures with no symptom of contamination were routinely indexed for the presence of cultivable bacterial and fungal contamination using the procedure developed by Kane (2000b).

**Callus Induction**

Leaves obtained from the in vitro shoot cultures served as explant sources for callus induction. Leaf explants were cut into 5 mm² sections with mid-vein, and the leaf margins removed with a scalpel. Leaf explants were cultured with abaxial surface in contact with the callus induction medium which were BM supplemented with TDZ at 0, 1, 5, 10 µM and 2,4-D at 0, 0.5, 1 µM. Explants were cultured in 100 × 15 mm sterile Petri plates containing 20 ml medium. There were 5 explants per Petri plate and 5 replicate plates per treatment. Cultures were initially maintained in dark for 8 weeks and then transferred to the 16 h light photoperiod at 40 µmol m⁻² s⁻¹ for another 4 weeks. The number of explants forming calli was scored after 12 weeks of culture. Callus formation frequency was calculated as the percentage of leaf explants forming calli.

**Indirect Shoot Organogenesis**

To evaluate medium for inducing shoot formation from callus, calli produced on callus induction medium containing 5 µM TDZ and 1 µM 2,4-D (the medium yielding optimal callus induction) were cultured on BM supplemented with factorial combinations of (1) 2iP at 0, 20, 40,
80 µM and IAA at 0 and 2 µM; (2) kinetin at 0, 1, 2, 4 µM and GA₃ at 0, 5, 10 µM. Plant growth regulator-free medium served as the control. Calli were separated from leaf explants after 12 weeks callus production. Calli were cut into small pieces with a fresh weight of approximately 150 mg, and then transferred to glass baby food jars (4.5 × 7 cm) containing 40 ml medium. There were 5 callus clumps per jar and 5 replicates per treatment. The number of shoots formed per callus was determined after 8 weeks culture under the 16 h photoperiod at 40 µmol m⁻² s⁻¹.

**Histological Analysis**

To verify the occurrence of indirect shoot organogenesis, callus samples were collected for histological examination 4 weeks after the initiation of culture and the 9 additional samples of 3 day intervals. For shoot differentiation, callus samples were taken every 3 days after callus clumps were transferred onto shoot induction medium until 42 days after culture. For optical light microscopy (OLM), the following procedure was used. Callus specimens weighting approximately 150 mg were fixed in Trumps fixative (McDowell and Trump, 1976). Fixative infiltration was achieved under vacuum until all samples of calli sank to the bottom of scintillation vials. Calli were rinsed 3 times in phosphate buffer (pH 7.2) for 10 min each. Calli were post-fixed in a 1% buffered osmium tetroxide solution for 1 h, and then rinsed 3 times in phosphate buffer (pH 7.2) for 10 min each, followed by 3 times wash in distilled water. Calli were dehydrated in a series of ascending aqueous ethanol solutions at 25%, 50%, 75% for 30 min each, at 95%, 100% for 1 h each, followed by dehydration in 100% acetone for 1 h. Calli were then embedded in Spur resin (Spurr, 1969). Callus sections (10 µm) were cut using a Leica Ultracut rotary ultramicrotome R (Leica Microscopy and Scientific Instruments, Deerfield, IL), and mounted on glass slides. Sections were stained with 0.2% toludine blue and examined under an Olympus BH-2 Epifluorescent Microscope (Olympus America Inc., Melville, NY). Photographs were taken using a Pixera 120C digital camera. For scanning electron microscopy
(SEM), callus samples of approximately 150 mg were immersed in 100% methanol. Samples were lyophilized using a Bal-Tec 030 critical point drier (ICMAS Inc., Alcoa, TN) with liquid CO₂, sputter coated with gold-palladium using a Denton Vacuum Desk II (Denton Vacuum, Moorestown, NJ) for approximately 50 s and viewed with a Hitachi S-4000 scanning electron microscope (Hitachi Scientific Instruments, Danbury, CT) operating at 6 KV. Digital images were processed using SEMages 16 software (Advance Database System, Inc., Denver, CO).

**Acclimatization**

Shoots, some with roots, were removed from the baby food jars and excised individually from the callus clumps. Medium was carefully rinsed off shoots. Shoots longer than 20 mm with 2-3 leaves were planted individually in 60-cell plug trays (4.5 × 4 × 5 cm³ each cell, REB Plastics Inc, Apopka, FL) containing a 2:1:1 (v/v) soilless mixture of Canadian peat: vermiculite: perlite. All plantlets were maintained in a greenhouse under shade cloth with a maximum irradiance of 345 µmol m⁻² s⁻¹, natural photoperiod (10 to 14.5 h light), and a temperature range of 20 to 31°C. Plugs were hand watered twice a week. Peters 20N-10P-20K liquid fertilizer (200 mg l⁻¹ N; The Scotts Company, Marysville, OH) was applied weekly following 2 weeks acclimatization.

**Statistical Analysis**

All experiments were established in a completely randomized design. The experiments showing treatment responses were repeated once. Data were subject to analysis of variance using SAS (SAS Institute, Inc., 1999). Mean separation was achieved by the least significant difference (LSD) test at 95% level.
Results

Callus Induction

The frequency of callus occurrence from medium containing TDZ and 2,4-D differed significantly based on their concentrations. No callus occurred on medium devoid of TDZ and 2,4-D or with 2,4-D only at 0.5 and 1.0 µM. However, TDZ alone at concentrations of 1, 5, and 10 µM induced 4, 10, and 26% of explants to produce calli respectively. TDZ was required for callus formation, but the higher frequency of callus formation occurred in medium supplemented with both TDZ and 2,4-D. Induction medium containing 5 µM TDZ and 1 µM 2,4-D resulted in the maximum of 96% of explants to produce calli (Table 2-1).

Indirect Shoot Organogenesis

A low number of shoots developed from calli cultured on BM alone (1.6 shoots/callus) or BM supplemented with 2 µM IAA (1.2 shoots/callus). Basal medium supplemented with 2iP alone at concentrations of 20, 40, and 80 µM elevated shoot numbers to 3.6, 6.3, and 4.0 per callus respectively. Combining 2iP with IAA further increased shoot numbers compared to 2iP at respective concentrations alone, but the increase was not statistically significant. Highest shoot number (7.9 shoots/callus) occurred on BM supplemented with 40 µM 2iP and 2 µM IAA (Table 2-2). No shoot formation was observed on BM supplemented with combinations of kinetin and GA3.

Green nodular calli were observed on leaf explants after 12 weeks of culture on BM supplemented with 5 µM TDZ and 1 µM 2,4-D (Figure 2-1A). When calli were separated from primary leaf explants and transferred onto BM supplemented with 40 µM 2iP and 2 µM IAA, small green meristems were visible on the surface of calli within 4 weeks (Figure 2-1B) and later developed into shoot buds (Figure 2-1C). Leaf formation and shoot elongation occurred in the
following 2 weeks (Figure 2-1D). Single shoot or shoot clusters with leaves and roots were
developed by the end of 8 weeks of culture (Figure 2-1E).

**Acclimatization**

*Dieffenbachia* cv. Camouflage plants were very easily acclimatized (Figure 2-1F). *Ex vitro* survival rate of 100% was observed.

**Histological Analysis**

Indirect shoot organogenesis was confirmed by histological sectioning. Calli were observed from leaf explants on BM medium supplemented with 5 µM TDZ and 1 µM 2,4-D after 28 days of culture. Two types of cells were observed in calli under light microscopy: regenerative cells which were smaller in size and more compact with more densely stained cytoplasm, thinner cell walls, more prominent nuclei and no visible vacuoles (Figure 2-2A); non-regenerative cells which were larger and less compact with less cytoplasm and smaller nuclei, thicker cell walls and larger vacuoles (Figure 2-2B). Early mitotic activity was observed after 31 days of culture (Figure 2-2C). The first cell division was usually anticlinal followed by periclinal cell division (Figure 2-2D). After several mitotic division, the differentiation of a meristematic zone occurred (Figure 2-2E). By continuous anticlinal and periclinal cell division, bigger meristematic cell masses composed of actively dividing cells were formed by 43 days culture. Each meristematic mass was characterized by cells with thick walls, and the individual cell was separated by thinner walls (Figure 2-2-F). Meristematic cell masses may also develop into globular shapes, assuming an appearance similar to globular somatic embryos (Figure 2-2G and 2-2H). Initial cell divisions usually were initiated from a superficial callus cell or cells (Figure 2-2D), but a cell or a group of cells in inner cell layers in the callus may also give rise to meristematic mass (Figure 2-2I). The meristematic mass became progressively more organized and formed a meristematic dome which represented an apical meristem of a bud after 12 days of
culture on shoot induction medium (Figure 2-2J). The cell division along the flanks of the bud meristem resulted in leaf primordia formation after 18 days shoot induction (Figure 2-2K). A well-developed adventitious bud with apical shoot meristem and leaf was formed after 27 days of culture (Figure 2-2L). Sometimes multiple shoots were formed (Figure 2-2M). Root formation occurred after 39 days of culture (Figure 2-2N). A complete plantlet was regenerated after 8 weeks culture on shoot induction medium. Vascular connection between a developing shoot and callus tissue was detected at day of 24 (Figure 2-2O). Scanning electron microscopy showed stomata were present on the epidermis of developing shoots at day 36 (Figure 2-2P).

**Discussion**

A plant regeneration system *via* indirect shoot organogenesis was established in this study. To our knowledge, this is the first report of indirect shoot organogenesis in *Dieffenbachia*. Our observations indicated that *Dieffenbachia* in general was recalcitrant in regard to shoot organogenesis or somatic embryogenesis (attempts were made to induce somatic embryogenesis failed to a wide range of PGR types, concentrations and combinations). This may, in part, explain why *in vitro* regeneration *via* organogenesis or somatic embryogenesis in *Dieffenbachia* has not been previously reported.

**Callus Induction and Shoot Formation**

The concentration and combination of PGR are the key factors influencing indirect shoot organogenesis in *Dieffenbachia*. A similar PGR effect on callus formation has been reported in other species (Khanam et al., 2000; Reddy et al., 2001; Ma and Xu, 2002; Giridhar et al., 2004; Azad et al., 2005; Datta and Majumder, 2005; Zhou and Brown, 2005). 2,4-D has been shown to be most effective for callus induction in many species. On the contrary, our study showed that 2,4-D was not a prerequisite for callus initiation as calli were induced on BM without 2,4-D. In contrast, TDZ was required for callus formation in *Dieffenbachia* cv. Camouflage. There have
been several reports of significant TDZ effects on callus formation and shoot organogenesis in other species (Gurriaran et al., 1999; Mithila et al., 2003; Datta and Majumder, 2005; Landi and Mezzetti, 2005). TDZ, a cotton defoliant, has both auxin-like and cytokinin-like activity and can be substituted for auxins or the combination of auxins and cytokinins (Singh et al., 2003). Hutchinson et al (1996) reported that TDZ could result in an increase in endogenous levels of auxins. It could be possible that TDZ might have fulfilled both auxin-like and cytokinin-like roles in callus induction of this *Dieffenbachia*.

Cytokinin type and concentration have significant effects on subsequent shoot regeneration from calli. Results from this study indicated that 2iP was more effective than kinetin because no shoot regeneration was observed on BM supplemented with kinetin alone or combination with GA₃. Voyiatzi and Voyiatzis (1989) also found that 2iP was more effective in inducing lateral shoot multiplication in *Dieffenbachia* than kinetin and 80 µM 2iP with 2 µM IAA was optimal for shoot formation of *D. exotica* cv. Marianna. The present results suggested that 40 µM 2iP with 2 µM IAA was optimal for shoot organogenesis of cv. Camouflage. This difference might be due to the fact that different cultivars, explants were used. This study used calli derived from leaves, while axillary buds and shoot tips were used for shoot multiplication of cv. Marianna (Voyiatzi and Voyiatzis, 1989). Differential responses of genotypes and explant sources to PGR requirements have been well documented (Magioli et al., 1998; Choi et al., 2003).

**Acclimatization**

*Dieffenbachia* shoots regenerated in vitro proved to be easily adaptable to ex vitro conditions. No separate in vitro rooting stage was required as shoot survival rate was 100% in a soilless substrate. This is different from many other species where rooting is an obstacle for plant establishment (Reed, 1995; Gavidia et al., 1996; Pruski et al., 2000). *Dieffenbachia* cv. Camouflage belongs to an easy-to-root type, which is in contrast to its very limited shoot
formation ability. It was conceivable that endogenous level of auxin in *Dieffenbachia* might be sufficient for root formation. A promotive carry over effect of the IAA in the shoot induction medium on rooting was also possible.

**Histological Analysis**

*In vitro* shoot organogenesis and somatic embryogenesis are the most used methods for a large-scale propagation and production of somaclonal variants. Sometimes it is very difficult to detect the true nature of *in vitro* regeneration especially when a callus phase is involved in the regeneration process and when the appearance of regenerating structures looks very much like somatic embryos. In our study, nodular calli were induced from cv. Camouflage. The appearance of these nodular calli superficially resembled globular somatic embryos and they also could be easily detached from leaf explants. When these nodular calli were separated from leaf explants and transferred onto BM medium supplemented with the combination of different plant growth regulators, they either degenerated or simply proliferated. Others (Haensch, 2004a, b; Salaj et al., 2005) have reported embryo-like structures were produced in their studies. However, histological analysis revealed in their studies that all these somatic embryo-like structures were composed of parenchymatous and vacuolated cells, and had no regeneration capacity. In contrast, histological examination in our study showed that these nodular calli consisted of actively dividing cells, therefore having regeneration potential. The reasons why these promising cells passed through organogenesis instead of somatic embryogenesis are not quite clear, but the fact that the route of morphogenesis could be changed by the manipulation of plant growth regulators in the culture medium has been demonstrated in other species. It seemed that we have not found an optimal combination of the genotype, explant tissue, type, concentration of plant growth regulators, or culture condition for inducing somatic embryogenesis in *Dieffenbachia*. It is worthy of further investigation. Apart from the influence of PGRs, adventitious shoot regeneration *in vitro* must
involve interrupting a genetically determined developmental pathway and reprogramming a new
developmental pathway at gene level (Schwarz and Beaty, 2000).

Our study indicated that we should evaluate critically publications on somatic
embryogenesis. More and more papers on somatic embryogenesis from different species have
been published in recent years. Some of these papers did not provide histological support, only
based on morphological similarities of regenerating structures with somatic embryos. Most of
them did complete a histological examination, but verifications of bipolar structures were not
provided. Usually shoot meristem and root meristem were shown in separate pictures. Only a
few publication presented histological evidence of bipolarity and the lack of vascular connection.

The ability to regenerate shoots from calli has several advantages. A great number of
shoots can be produced from an explant through callus induction and shoot formation. Indirect
shoot organogenesis has a greater potential for regenerating somaclonal variants. In
*Dieffenbachia* cv. Camouflage, phenotypic variations in leaf variegation and color of
acclimatized plants were observed. The feasibility of inducing indirect shoot organogenesis in
other *Dieffenbachia* cultivars, and then evaluating this regeneration protocol for potential
isolation of somaclonal variants will be described in Chapter 3 and 4.
Table 2-1. Effect of TDZ and 2,4-D supplementation to BM on frequency of callus formation from leaf explants of *Dieffenbachia* cv. Camouflage cultured for 8 weeks in dark and 4 weeks in a 16 h light photoperiod at 40 µmol m$^{-2}$ s$^{-1}$.

<table>
<thead>
<tr>
<th>TDZ (µM)</th>
<th>2,4-D (µM)</th>
<th>Shoots/callus ± SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0 a</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>0 a</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>0 a</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>4 ± 0.1 a</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>46 ± 0.3 b</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>66 ± 0.2 c</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>10 ± 0.3 a</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>60 ± 0.3 c</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>96 ± 0.1 d</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>26 ± 0.3 e</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>76 ± 0.2 f</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>78 ± 0.2 f</td>
</tr>
</tbody>
</table>

* Means followed by the same letter are not significant at 0.05 level. Data represent means of two repeated experiments, each with 5 replicates and 5 subsamples per replicate.
Table 2-2. Effect of 2iP and IAA concentrations and combinations on shoot regeneration from *Dieffenbachia* cv. Camouflage calli cultured for 8 weeks in a 16 h light photoperiod at 40 µmol m$^{-2}$ s$^{-1}$.

<table>
<thead>
<tr>
<th>2iP (µM)</th>
<th>IAA (µM)</th>
<th>Shoots/callus ± SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.6 ± 0.3 a</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>1.2 ± 0.4 a</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>3.6 ± 0.9 b</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>4.2 ± 2.0 b</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>6.3 ± 2.2 c</td>
</tr>
<tr>
<td>40</td>
<td>2</td>
<td>7.9 ± 1.5 c</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>4.0 ± 0.1 b</td>
</tr>
<tr>
<td>80</td>
<td>2</td>
<td>3.8 ± 0.4 b</td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not significant at 0.05 level. Data represent means of two repeated experiments, each with 5 replicates and 5 subsamples per replicate.
Figure 2-1. Indirect shoot organogenesis in *Dieffenbachia* cv. Camouflage. A) Induction of calli on leaf explants on BM supplemented with 5 µM TDZ and 1 µM 2,4-D after 8 weeks culture in dark and 4 weeks culture in a 16 h light photoperiod. Bar = 1 mm. B) Small green meristems formed on the surface of calli on BM containing 40 µM 2iP and 2 µM IAA within 4 weeks. Bar = 1mm. C) Shoot buds formed from these meristems 2 weeks later. Bar = 1 cm. D) Shoots and leaf development. Bar = 1 cm. E) Well-developed shoots with roots within 8 weeks of culture. Bar = 1 cm. F) Acclimatized plants in the greenhouse exhibiting variation in leaf variegation and color. Bar = 5 cm.
CHAPTER 3
FACTORS AFFECTING INDIRECT SHOOT ORGANOGENESIS IN *Dieffenbachia*

**Introduction**

*Dieffenbachia*, a monocot belonging to the family Araceae, noted for its attractive, striking foliage, is one of the most important ornamental plant genera (Chen et al., 2004). Traditional breeding in *Dieffenbachia* can be hampered by its naturally-occurring dichogamy, long breeding cycle, poor seed production and germination (Henny, 1988). Selection of somaclonal variants from *in vitro* regenerated populations is an alternative means for new cultivar development (Henny and Chen, 2003). Due to the intervening callus phase, regeneration through indirect shoot organogenesis has been shown to produce more somaclonal variants (Chen et al., 2003a). Selection and subsequent *in vitro* propagation of variants with desired phenotypes can facilitate the release of new cultivar in 2-3 years compared to 7-10 years through the traditional breeding.

Among factors influencing *in vitro* regeneration *via* indirect shoot organogenesis, genotype, explant source, and plant growth regulators in media are the most important. Genotypic differences in shoot organogenesis have been observed in a wide range of species. Some genotypes exhibit high regenerative capacity, while others are either recalcitrant or exhibit no capacity at all (Kuehnle and Sugil, 1991; Nontaswatsri et al., 2002). Explant type may also influence capacity for in indirect shoot organogenesis, which may be related to the totipotency of cells at their developmental stages (Agarwal and Ranu, 2000; Bacchetta et al., 2003). Additionally, the type, concentration and combination of plant growth regulators in the media can greatly affect callus formation and subsequent shoot induction (Mithila et al., 2003; Thao et al., 2003).

To date, successful *in vitro* regeneration in *Dieffenbachia* has been largely limited to shoot culture from shoot tip or axillary bud explants (Knauss, 1976; Voyviation and Voyiatzis, 1989).
We have established a protocol for indirect shoot organogenesis in *Dieffenbachia cv. Camouflage* (Chapter 2), but information on the effects of genotype, explant source and plant growth regulators on callus-mediated shoot organogenesis in *Dieffenbachia* cultivars is lacking. The objectives of this study were to: 1) examine differences in capacity for indirect shoot organogenesis in 4 *Dieffenbachia* cultivars and 2) investigate the effects of explant type and plant growth regulators (type, concentration and combination) on indirect shoot organogenesis. It is expected that information obtained from this study will help establish a more efficient protocol for new cultivar development in *Dieffenbachia* through selection of somaclonal variants.

**Materials and Methods**

**Plants Materials, Media and Culture Conditions**

*In vitro* shoot cultures of the 4 *Dieffenbachia* cvs. Camouflage, Camille, Octopus and Star Bright served as the explant source. Previous genetic analysis demonstrated that these cultivars are genetically different (Chen et al., 2004). For callus induction and shoot organogenesis, a basal medium (BM) consisting of MS (Murashige and Skoog, 1962) mineral salts, 0.4 mg l⁻¹ thiamine, 2.0 mg l⁻¹ glycine, 100.0 mg l⁻¹ myo-inositol, 0.5 mg l⁻¹ pyridoxine, 0.5 mg l⁻¹ nicotinic acid, and 25 g l⁻¹ sucrose supplemented with plant growth regulators was used in all experiments. Medium was adjusted to a pH of 5.8 with 0.1 N KOH prior to the addition of 6 g l⁻¹ TC agar (PhytoTechnology Laboratories, Shawnee, Mission, KS) and autoclaving at 1.2 kg cm⁻² for 20 min. Cultures were maintained either in dark or under a 16 h light photoperiod at 40 µmol m⁻² s⁻¹ provided by cool white fluorescent lamps (General Electric F20WT12-CW) at 22 ± 3ºC.

**Callus Induction**

Leaves and roots from 4 week-old *in vitro* shoot cultures of the 4 *Dieffenbachia* cultivars Camouflage, Camille, Octopus and Star Bright served as explants. Leaves were cut into 5 mm² sections with mid-vein, and the leaf margins removed with a scalpel. Leaf explants were cultured
with the abaxial surface in contact with the callus induction media. Roots were cut into 10 mm long segments, and then cultured horizontally on media. A series of 8 screening experiments were completed to select the most effective concentration and combination of PGRs. Each experiment consisted of factorial PGR combination with PGR-free medium serving as the control: (1) BA at 0, 1, 10, 50 and 2,4-D at 0, 1, 10, 50 µM; (2) CPPU at 0, 1, 2.5, 5 and 2,4-D at 0, 2, 4, 8, 10 µM; (3) CPPU at 0, 1, 2.5, 5 NAA at 0, 2, 4, 8, 10 µM; (4) kinetin at 0, 1, 5, 10 and IAA at 0, 1 µM; (5) dicamba at 0, 1, 3, 9 and 2,4-D at 0, 1 µM; (6) picloram at 0, 1, 3, 9 and 2, 4-D at 0, 1 µM; (7) TDZ at 0, 1, 10, 50 and NAA at 0, 1, 10, 50 µM; (8) TDZ at 0, 1, 5, 10 and 2,4-D at 0, 0.5, 1 µM. Explants were cultured in 100 × 15 mm sterile Petri plates containing 20 ml medium. There were 5 explants per Petri plates and 5 replicate plates per treatment. Petri plates were sealed with one layer of Nescofilm (Karlan Research Products Corp., Cottonwood, AZ). Cultures were initially maintained in dark for 8 weeks for all cultivars except Star Bright that were in dark for 12 weeks (no any response observed by 8 weeks culture) and then transferred to a 16 h light photoperiod for another 4 weeks. The number of explants forming calli was scored after 12 weeks culture for cvs. Camouflage, Camille, Octopus and 16 weeks for Star Bright. The frequency of callus formation was calculated as the percentage of leaf explants forming calli.

**Shoot Induction**

Calli were excised from leaf explants, cut into pieces with a fresh weight of approximately 150 mg and then transferred to the shoot induction medium which was BM supplemented with 80 µM 2iP and 2 µM IAA. Calli were cultured in baby food jars containing 40 ml media with 5 callus clumps per vessel and 5 replicates per treatment. Culture vessels were sealed with one layer of Nescofilm. The number of shoots formed per callus was determined after 8 weeks culture under a 16 h light photoperiod.
**Acclimatization**

Shoots, some with roots, were removed from the vessels and excised individually from the callus clumps. Medium was carefully rinsed off the shoots. Shoots longer than 20 mm with 2-3 leaves were planted individually in 60-cell plug trays (cell dimensions: 4.5 × 4 × 5 cm³, REB Plastics Inc, Apopka, FL) containing a 2:1:1 (v/v) soilless mixture of Canadian peat: vermiculite: perlite. All plantlets were maintained in a shaded greenhouse under natural photoperiod (10 to 14.5 h light) with a maximum irradiance of 345 µmol m⁻² s⁻¹ and a temperature range of 20 to 31°C. Plugs were hand watered twice a week. Peters 20N-10P-20K liquid fertilizer (200 mg/l N; The Scotts Company, Marysville, OH) was applied weekly.

**Statistical Analysis**

All experiments were established in a completely randomized design. Experiments showing responsive treatments were repeated once. Data were subject to analysis of variance using SAS (SAS Institute, Inc., 1999). Mean separation was achieved by least significant difference (LSD) test at the 95% level.

**Results**

**Explant Effects**

A distinct difference in callus formation was observed between leaf and root explants. No callus was induced on root explants regardless of cultivar and PGR combination. However callus formation occurred on leaf explants of all the 4 cultivars cultured on BM containing TDZ and 2,4-D at appropriate concentrations.

**Genotypic Effects**

Leaf explants of different cultivars exhibited different responsiveness for callus formation. Callus initiation occurred from the leaf margins of cvs. Camouflage, Camille and Octopus after 4 weeks of culture; but no callus was noted on cv. Star Bright explants until 8 weeks culture. Leaf
explants first exhibited elongation and expansion, then became curved and swollen prior to callus proliferation.

The effect of genotype on callus morphology was evident. Four morphologically distinct callus types varied in structure and color were observed. Green nodular (Figure 3-1A), brown nodular (Figure 3-1B), yellow friable, mucilaginous (Figure 3-1C) and light green compact (Figure 3-1D) calli were produced from leaf explants of cvs. Camouflage, Camille, Octopus and Star Bright respectively. Based on visual observation, callus growth rate also differed among cultivars. Octopus produced the most calli covering almost the entire surface of the leaf explants. Least calli was produced on Star Bright with callus production only limited to the cut ends of leaf explants. Moderate growth rate of calli was observed in cvs. Camouflage and Camille.

Differences in the frequency of callus formation among cultivars were significant. Maximum frequency of callus formation (96%) was obtained from Camouflage leaf explants, followed by 66% from Octopus, 62% from Camille and 52% from Star Bright on BM supplemented with 5 µM TDZ and 1 µM 2,4-D (Table 3-1).

A significant effect of genotype on shoot induction was also observed. After calli were transferred onto BM supplemented with 80 µM 2iP and 2 µM IAA, maximum shoot production (6.7 shoots/callus) was obtained from cv. Camouflage, followed by 4.4 shoots/callus from cv. Camille, 3.5 shoots/callus from cv. Star Bright. Cultivar Octopus displayed no capacity for indirect shoot regeneration (Table 3-1).

**PGR Effects**

PGR type, concentration and combination had significant effects on callus induction on cultured leaf explants. Among the PGRs screened, callus formation was only observed on leaf explants cultured on BM supplemented with factorial combinations of 0, 1, 5, 10 µM TDZ and 0.5 and 1 µM 2,4-D. Other PGRs or their combinations failed to induce callus formation from

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50
either leaf or root explants. Additionally, the effect of TDZ and 2,4-D on the frequency of callus formation varied significantly. No callus could be induced on the medium without any PGRs. TDZ was required for callus induction for cvs. Camouflage, Camille and Star Bright. No callus formation was observed on media without TDZ in these 3 cultivars. For cv. Octopus, a 2% frequency of callus formation was noted on medium without TDZ. However, 2,4-D was required for callus production on Camille explants. Although callus formation was observed on media without 2,4-D for the other 3 cultivars, the frequency of callus formation was lower (4% to 26%). Callus production increased with increasing 2,4-D concentrations. Consequently, uniformed callus formation was obtained on BM supplemented with both TDZ and 2,4-D for all the 4 cultivars. In general callus formation was promoted with increasing levels of both TDZ and 2,4-D, but high TDZ concentration (≥ 10 µM) inhibited callus formation (Data not shown). The combination of 5 µM TDZ and 1 µM 2,4-D was optimal for callus production among the 4 cultivars tested (Table 3-1).

A distinct carry-over effect of TDZ and 2,4-D from callus induction media on subsequent shoot organogenesis was noted. Calli produced on callus induction medium with higher levels of 2,4-D displayed reduced capacity for shoot development. Conversely, calli produced on medium supplemented with higher levels of TDZ were more highly shoot organogenic (Table 3-1). When cultured on BM supplemented with 80 µM 2iP and 2 µM IAA, calli derived from BM supplemented with 10 µM TDZ alone exhibited the highest shoot regeneration (6.7 ± 1.3 shoots/callus) in cv. Camouflage.

The developmental process of indirect shoot organogenesis was illustrated in cv. Camille. Brown nodular calli were produced on leaf explants of cv. Camille on BM supplemented with 5 µM TDZ and 1 µM 2,4-D after 8 weeks in dark and 4 weeks cultures in a 16 h light photoperiod.
(Figure 3-2A). After calli were separated from leaf explants and transferred onto BM supplemented with 80 µM 2iP and 2 µM IAA for shoot induction, small green meristems appeared on the surface of calli within 4 weeks (Figure 3-2B). Shoot buds developed from these meristems by the 6th week (Figure 3-2C). Leaf formation and shoot elongation occurred within 8 weeks (Figure 3-2D). Shoot clusters with well-developed leaves and roots were formed by the end of 8 weeks culture (Figure 3-2E).

**Acclimatization**

Shoot and root formation was observed in most *Dieffenbachia* cultures. Shoots longer than 20 mm with 2 to 3 leaves were easily acclimatized in a shaded greenhouse (Figure 3-2F). *Ex vitro* survival 100% was obtained in all the cultivars acclimatized.

**Discussion**

The present study demonstrates the difficulties in the induction of indirect shoot organogenesis in *Dieffenbachia*. Attempts to use leaf explants taken directly from plants grown in the greenhouse to initiate callus production failed due to significant (> 70%) culture contamination. Leaf explants obtained from greenhouse plants displayed no response regardless of media tested. We demonstrated that using leaf explants from *in vitro*-produced shoots was a viable, alternative method to reduce contamination and increase explant responsiveness. However, the responsiveness of leaf explants from *in vitro* plants on callus induction media was slow. At least 12 weeks (16 weeks for cv. Star Bright) were required for callus induction. The shoot regeneration capacity of *Dieffenbachia* from callus was low with a maximum 6.3 shoots/callus. Slow responsiveness to PGR treatments, high contamination rate *in vitro* and recalcitrant nature for *in vitro* culture may explain why indirect shoot organogenesis has not been reported in *Dieffenbachia* by others.
Other factors may account for high contamination rate. *Dieffenbachia* is mainly propagated vegetatively by cuttings and divisions, and is maintained under shaded condition which may allow bacteria and fungi to accumulate on the plant surface. The presence of endophytes can not be ruled out (Knauss, 1976). This might be one reason for such high contamination rate in our attempts to establish *in vitro* culture. Additionally, *Dieffenbachia* is a naturally-slow- growing plant and this may also be manifested *in vitro*.

**Genotype Effects**

*Dieffenbachia* is a monocotyledonous genus and is among the most recalcitrant plants for regeneration. In the present study, 4 cultivars evaluated are of different genetic origin. Camille, one of the most popular cultivars in the foliage plant industry, is a sport selected from cv. Perfection, while cv. Star Bright is an interspecific hybrid selected from crosses of several parents. Camouflage was selected from somaclonal variants of cv. Panther, while Octopus was isolated from somaclonal variants of Camouflage (Chen et al., 2004).

Genotype remains a key determinant of capacity for indirect shoot organogenesis. There were distinct differences in callus morphology, callus forming ability and subsequent shoot differentiation among the 4 *Dieffenbachia* cultivars. In the present study, 4 morphologically distinct types of calli have been observed. Among them, three types were organogenic and one type non-organogenic. The frequency of callus formation was also genotype-dependent with the highest (96%) observed in cv. Camouflage. Genotypic differences were still obvious after calli were transferred onto shoot induction medium. Calli of cv. Octopus did not exhibit any shoot formation while Camouflage exhibited the highest shoot formation (6.7 shoots/callus).

Variation in callus morphology and callus forming ability of different genotypes has been previously reported in other species (Thao et al., 2003; Landi and Mezzetti, 2005). Our results were consistent with these reports. Kallak et al (1997) concluded that callogenesis was not only a
result of dedifferentiation of explant tissues, but also an essential preparatory stage for in vitro morphogenesis and callus type may be an indicator for shoot organogenic potential. In their analysis of genetic relatedness of *Dieffenbachia* cultivars using AFLP, Chen et al (2004) found that cv. Camouflage significantly differed from cvs. Camille, Octopus and Star Bright as it was positioned in one genetic cluster and the other three shared another cluster. This might partially explain why cv. Camouflage performed differently in terms of the frequency of callus formation and shoots/callus compared to the other 3 cultivars. Phillips (2004) reported that specific genes involved in each stage of shoot organogenesis (dedifferentiation, induction and differentiation). In some genotypes genes involved in shoot organogenesis may be suppressed due to inappropriate culture condition.

**PGR Effects**

The type, concentration and combination of PGRs in media are another key factor regulating shoot organogenesis. The present study showed that PGRs have significant effects on the induction of calli. 2,4-D has been shown to be the most effective for callus induction in a variety of species (Ma and Xu, 2002; Thao et al., 2003). In contrast, we observed that 2,4-D was not required for callus initiation in 3 of 4 cultivars. Among PGRs screened, TDZ was most effective in stimulating callus formation. TDZ has also been found effective in in vitro regeneration in a variety of species, such as pelargonium (Haensch, 2004b), African violet (Mithila et al., 2003), geranium (Robichon et al., 1997). Hutchinson et al (1996) reported that TDZ treatment could result in an increase in endogenous auxin levels. This may explain why TDZ was most effective in callus induction in present study because auxins usually stimulate callus formation.

TDZ also has cytokinin-like activity (Zhang et al., 2001; Landi and Mezzetti, 2006). Induction of indirect shoot organogenesis in *Dieffenbachia* cultures supports this claim. It was
noted that many small shoot meristems formed on the surface of calli on media with TDZ, but these shoots did not elongate. Attempt to include GA3 in media to elongate these stunted shoots failed. Bacchetta et al (2003) reported that TDZ induced multiple shoots with stunted growth in Lilium. Orlikowska et al (1995) noted 4.5 µM TDZ in combination with 5.4 µM NAA induced direct shoot organogenesis on petiole explants in an unidentified *Dieffenbachia* cultivar.

**Carry-Over Effect of PGRs**

Depending on the type and concentration, PGRs in the callus induction media may have had either a negative or positive carry-over effect on subsequent shoot formation (Vardja and Vardja, 2001). In our study, we observed that shoot forming ability was higher from calli cultured on media containing TDZ alone. Similar result was found in indirect shoot organogenesis in *Duboisia myoporoides* in which the combination of cytokinin/auxin used for callus induction had effect on subsequent shoot induction (Khanam et al., 2000). Calli derived from TDZ-supplemented media may have accumulated TDZ or a metabolite in their cells during the period of culture. When these calli were transferred to shoot induction media, high TDZ levels in callus cells may have stimulated shoot proliferation.

**Explant Effects**

The effect of explant sources on indirect shoot organogenesis has been reported by other authors (Orlikowska et al., 1995; Kallak et al., 1997; Berrios et al., 1999; Mithila et al., 2003). This study showed that only leaf, not root explants, exhibited the capacity for indirect shoot organogenesis. Root explants failed to indirect shoot organogenesis. An explanation for this difference would be that cells from root explants were organogenically less competent than those of leaf explants. Another possible reason could be that PGR combinations and/or their concentrations screened were inappropriate for callus induction on root explants. Orlikowska et al (1995) also reported that *Dieffenbachia* root explants were non-regenerative *in vitro.*
Acclimatization

In contrast to their recalcitrant nature for callus induction and shoot regeneration, microcuttings of *Dieffenbachia* cultivars were easy-to-root and 100% *ex vitro* survival of shoots was achieved in all the 3 cultivars exhibiting the capacity for indirect shoot organogenesis. Root formation occurred concurrently with shoot formation on shoot induction medium. It was conceivable that endogenous auxin levels in *Dieffenbachia* microcuttings were sufficient for root formation. Variations in leaf variegation and color (in cv. Camouflage) and leaf morphology (in cv. Camille) were observed among acclimatized plants. The evaluation of this regeneration protocol for potential isolation of somaclonal variants will be described in Chapter 4.
Figure 3-1. Characterization of calli cultured on BM supplemented with 5 μM TDZ and 1 μM 2,4-D after 8 weeks culture in dark for cvs. Camouflage, Camille and Octopus (12 weeks for cv. Star Bright) and 4 weeks culture in a 16 h light photoperiod. A) Green nodular calli of cv. Camouflage. Bar = 1 mm. B) brown nodular calli of cv. Camille. Bar = 1 mm. C) Yellow, friable calli of cv. Octopus. Bar = 5 mm. D) green compact calli of cv. Star Bright. Bar = 5 mm.
Table 3-1. Effects of TDZ and 2,4-D on the frequency of callus formation on leaf explants and shoot number per callus of *Dieffenbachia* 4 cultivars Camouflage, Camille, Octopus and Star Bright.

<table>
<thead>
<tr>
<th>TDZ (µM)</th>
<th>2,4-D (µM)</th>
<th>Camouflage</th>
<th>Camille</th>
<th>Octopus</th>
<th>Star Bright</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Callus formation frequency (% ± SE)</td>
<td>Shoots/callus ± SE</td>
<td>Callus formation frequency (% ± SE)</td>
<td>Shoots/callus ± SE</td>
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<tr>
<td>0</td>
<td>0.5</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td>2 ± 0.2 a</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>4 ± 0.1 a</td>
<td>0 a</td>
<td>0 a</td>
<td>6 ± 0.3 a</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>46 ± 0.3 b</td>
<td>3.7 ± 0.5 b</td>
<td>8 ± 0.2 a</td>
<td>0 a</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>66 ± 0.2 c</td>
<td>2.7 ± 0.5 c</td>
<td>28 ± 0.2 bd</td>
<td>0 a</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>10 ± 0.3 a</td>
<td>0 a</td>
<td>0 a</td>
<td>14 ± 0.4 ab</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>60 ± 0.3 c</td>
<td>4.4 ± 0.5 b</td>
<td>22 ± 0.2 b</td>
<td>0 a</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>96 ± 0.1 d</td>
<td>3.5 ± 0.5 bc</td>
<td>62 ± 0.3 c</td>
<td>3.7 ± 0.5 b</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>26 ± 0.3 e</td>
<td>6.7 ± 1.3 d</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>76 ± 0.2 f</td>
<td>5.3 ± 0.7 e</td>
<td>36 ± 0.3 d</td>
<td>0 a</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>78 ± 0.2 f</td>
<td>3.8 ± 0.4 b</td>
<td>56 ± 0.3 c</td>
<td>4.4 ± 0.5 c</td>
</tr>
</tbody>
</table>

Callus formation frequency was evaluated after 8 weeks for cv. Camouflage, Camille and Octopus (12 weeks for cv. Star Bright) in dark and 4 weeks in a 16 h light photoperiod on BM supplemented with different combination of TDZ and 2,4-D. Shoots per callus were scored after 8 weeks in a 16 h light photoperiod on BM supplemented with 80 µM 2iP and 2 µM IAA.
Figure 3-2. Indirect shoot organogenesis in *Dieffenbachia* cv. Camille. A) Induction of calli on leaf explants on BM supplemented with 5 µM TDZ and 1 µM 2,4-D after 8 weeks culture in dark and 4 weeks culture in a 16 h photoperiod. Bar = 500 µm. B) Small green meristems formed on the surface of calli on BM containing 80 µM 2iP and 2 µM IAA within 4 weeks. Bar = 1cm. C) Shoot buds formed from these meristems by the 6th week. Bar = 1cm. D) Leaf formation and shoot elongation occurred. Bar = 1cm. E) Well-developed shoots with roots within 8 weeks of culture. Bar = 1 cm. F) Acclimatized plants in the greenhouse. Bar = 1 cm.
CHAPTER 4
ASSESSMENT OF SOMACLONAL VARIATION IN *Dieffenbachia* PLANTS
REGENERATED FROM INDIRECT SHOOT ORGANOGENESIS AT PHENOTYPIC LEVEL

Introduction

Phenotypic variation observed among plants regenerated from tissue culture is referred to as somaclonal variation (Larkin and Scowcroft, 1981). Somaclonal variation can be assessed by analysis of phenotype, chromosome number and structure, proteins, or direct DNA evaluation of plants (Bouman and De Klerk, 1997). The extent of phenotypic variation is usually determined as the percentage of plants showing aberrations from the parental plant for one or more defined characteristics. These characteristics may include change in foliar variegation pattern, modification of flower color and size, alteration of leaf shape, increase in lateral shoots, and change in overall plant form (Griesbach et al., 1988; Khalid et al., 1989). Plants with the deviant phenotypes are known as somaclones or somaclonal variants. Commercially, visual phenotypic evaluation is paramount since the value of the plants, especially floriculture crops, lies in appearance. Chen and Henny (2006) documented the occurrence of somaclonal variation in 58 genera across 33 families of floriculture crops and proposed that somaclonal variation is an important source for cultivar development of floriculture crops.

Many factors, including genotype, growth regulators, and tissue source are involved in somaclonal variation (Karp, 1991; Chen and Henny, 2006). Among these factors, plant genotype is probably the most important determinant of variation. Some cultivars show higher variation rate while others are highly stable (Najaran and Walton, 1987; Skirvin et al., 1994; Bouman and de Klerk, 1997). In addition, the duration of tissue culture also affects somaclonal variation. Somaclonal variation generally increases with the time that a culture has been maintained *in vitro*, especially for callus culture; the longer a culture remains *in vitro*, the greater somaclonal variation (Orton, 1985; Rodrigues et al., 1998).
Dieffenbachia belongs to the family Araceae and consistently ranks among the top five most popular ornamental foliage plant genera. Due to its naturally occurring dichogamy, limited seed production, development of a new Dieffenbachia cultivar through traditional breeding usually requires 7 to 10 years (Henny and Chen, 2003). With the establishment of in vitro culture techniques for Dieffenbachia propagation in the 1980s, new cultivars have been selected from somaclonal variants and released in the foliage plant industry (Chen and Henny, 2006).

However, there is no report on the evaluation of somaclonal variation among Dieffenbachia plants regenerated from indirect shoot organogenesis.

Our previous studies established methods for regenerating the 3 Dieffenbachia cultivars Camouflage, Camille, and Star Bright through indirect shoot organogenesis (Chapter 3). The objectives of the present study were to phenotypically assess somaclonal variation among the regenerants of the 3 Dieffenbachia cultivars and determine the effects of genotype and the duration of callus culture on shoot regeneration and somaclonal variation.

Materials and Methods

Regeneration of the 3 Dieffenbachia cultivars Camouflage, Camille, and Star Bright through indirect shoot organogenesis was described in Chapter 3, which can be briefly outlined as follows:

Callus Induction

Leaves obtained from in vitro shoot culture of the 3 cultivars were cut into 5 mm² sections containing mid-vein. A callus induction medium consisted of a basal medium (BM) containing MS (Murashige and Skoog, 1962) mineral salts, 0.4 mg l⁻¹ thiamine, 2.0 mg l⁻¹ glycine, 100.0 mg l⁻¹ myo-inositol, 0.5 mg l⁻¹ pyridoxine, 0.5 mg l⁻¹ nicotinic acid, and 25 g l⁻¹ sucrose which was supplemented with 5 µM TDZ and 1 µM 2,4-D. Medium was adjusted to pH 5.8 with 0.1 N KOH prior to the addition of 6 g l⁻¹ TC agar (PhytoTechnology Laboratories, Shawnee Mission,
KS) and autoclaved at 1.2 kg cm\(^{-2}\) for 20 min. Leaf explants were cultured in 100 × 15 mm sterile Petri plates containing 20 ml medium. There were 5 explants per plate inoculated with abaxial surface in contact with the medium. The culture was initially maintained in the dark for 8 weeks for cvs. Camouflage and Camille, and 12 weeks for Star Bright (due to no responses by 8 weeks culture), and then transferred to a 16 h light photoperiod at 40 μmol m\(^{-2}\) s\(^{-1}\) provided by cool white fluorescent lamps (General Electric F20WT12CW) at 22 ± 3°C for another 4 weeks.

**Sustained Callus Culture**

Calli were excised from leaf explants at the end of callus induction, and cut into pieces weighing approximately 150 mg (fresh weight). Half the calli were transferred to the same fresh callus induction medium for sustained callus production. The other half was transferred to shoot induction medium to determine effects of subculture number on the capacity for shoot regeneration from calli. Calli were subcultured at 8 week intervals by this manner.

**Shoot Induction**

Five callus pieces, each weighing approximately 150 mg, were transferred into individual baby food jar containing 40 ml shoot induction medium. The shoot induction medium was composed of BM supplemented with 40 μM 2iP and 2 μM IAA and solidified with 6 g l\(^{-1}\) TC agar. Cultures were maintained under a 16 h light photoperiod with a light intensity of 40 μmol m\(^{-2}\) s\(^{-1}\) at 22 ± 3°C for 8 weeks. The number of shoots per callus piece was recorded for each subculture.

**Acclimatization**

After 8 weeks on shoot induction medium, shoots, some with roots, were removed from the vessels and excised individually from the callus clumps. Medium was carefully rinsed from the shoots. Shoots longer than 20 mm with 2-3 leaves were planted individually in 60-cell plug trays (cell dimensions: 4.5 × 4 × 5 cm\(^3\), REB Plastics Inc, Apopka, FL) containing a 2:1:1 (v/v)
soilless mixture of Canadian peat: vermiculite: perlite. All plantlets were maintained in a shaded greenhouse under natural photoperiod (10 to 14.5 h light) with a maximum irradiance of 345 µmol m⁻² s⁻¹ and a temperature range of 20 to 31°C. Plugs were hand watered twice a week. Peters 20N-10P-20K liquid fertilizer (200 mg/l N; The Scotts Company, Marysville, OH) was applied weekly following 2 weeks acclimatization.

**Determination of Somaclonal Variation**

Plants grown in plug trays were regularly checked for the presence of variant phenotypes. Emphasis was primarily placed on foliar characteristics as *Dieffenbachia* is valued for its leaf variegation. Novel variegation patterns were described, photographed, and grouped into different types of somaclonal variant. Numbers of plants per variant type were counted, and percentage of such type in relation to the total number of regenerated plants was calculated.

Parental and variant types of plants regenerated from *Dieffenbachia* cvs. Camouflage and Camille were then transplanted from plug trays to 15-cm diameter plastic pots containing Vergo Container Mix A (Verlite Co. Tampa, FL). The components of the substrate were 2:1:1 (v/v) of Canadian peat: vermiculite: perlite. Five grams of an 18.0N-2.6P-10.0K controlled-released fertilizer with microelements (Multicote 18-6-12, Haifa Chemicals Ltd., Haifa Bay, Israel) was applied to the soil surface in each pot. Plants were grown on raised benches in a shaded and evaporated pad cooled greenhouse under a maximum photosynthetically active radiation of 350 µmol m⁻² s⁻¹. Temperatures ranged from 20 to 30°C and relative humidity from 50 to 100%. Plants were overhead irrigated through sprinklers one to two times a week. Plant height, canopy height and width, length and width of the largest leaf, number of basal shoots were recorded 8 months after growing in the shaded greenhouse.

Both shoot and cane cuttings were then made from parental plants and identified variants and rooted in Vergo Container Mix A in the aforementioned shaded greenhouse. Morphological
characteristics of cutting-propagated progenies were compared to respective parents for determining if the variant phenotypes were stable.

**Examination of the Duration of Callus Culture Affecting Somaclonal Variation**

Two populations of regenerated plants of *Dieffenbachia* cv. Camouflage were used to examine the duration of callus culture phase influencing somaclonal variation. One population was derived from 8 months callus culture *in vitro*. The other population was derived from 16 months callus culture *in vitro*. Plants with novel foliar variegation patterns were grouped as mentioned above. Numbers of plants per variant type were counted and calculated in relation to the total number of regenerated plants in each population.

**Experimental Design and Statistical Analysis**

All experiments in this study were established in a completely randomized design. Data for plant growth were subject to analysis of variance using SAS (SAS institute Inc., 1999). Least-squares mean test was used to compare means at the 95% level of probability.

**Results**

**Somaclonal Variant Identification**

A total of 2,248 plants from cv. Camouflage, 384 of cv. Camille and 62 of cv. Star Bright were regenerated from indirect shoot organogenesis. Novel phenotypes were observed from regenerants of cvs. Camouflage and Camille, but not from cv. Star Bright. Three types of variants, called SV1, SV2, and SV3, were identified among regenerated Camouflage plants; each had distinct phenotype (Figure 4-1). The parental plant had camouflaged leaves with random green batches. Leaves of SV1, however, were green with whitish variegation along the midvein. SV2 had light green leaves with many yellowish spots, and connections among the spots resulted in large yellowish blotches. Leaf color of SV3 was similar to SV2 but had fewer yellowish spots. The other differences between SV2 and SV3 were that SV2 had some white spots on leaves and
sparkled petioles. The leaf shape of the 3 variants was comparable to that of their parent. In addition to these morphological differences, analysis of variance for plant growth parameters showed that SV1 significantly differed in plant height, canopy height and width, leaf length from the parental plant and SV2. SV1 was taller with larger canopy and longer leaves than parental plants and SV2. SV2 and SV3 had no basal shoots (single stem) but basal shoot numbers between SV1 and parental plants were similar ranging from 3 to 4 (Table 4-1). No statistical analysis could be done with SV3 because only 1 plant was available.

One variant type was identified among the regenerants of cv. Camille. This variant had the same foliar variegation pattern as its parent but possessed lanceolate leaves compared to the oblong leaves of the parent (Figure 4-2). The ratio of leaf length and leaf width was 3.4 for the variants compared to 1.5 for parental plants. The variant also exhibited significant difference from the parent in growth parameters measured except for basal shoot number. Variants grew taller with larger canopy and had longer, narrower leaves than the parent (Table 4-2). The morphological characteristics of all variants, 3 types from cv. Camouflage and 1 from cv. Camille, were stable in shoot and cane cutting propagated population.

**Genotype Differences**

Among the 2,248 regenerated plants of cv. Camouflage, a total of 908 variant plants were isolated, of which 904 plants belonged to the SV1 type, 3 plants belonged to the SV2 type, and one being the SV3 type. Correspondingly, the occurrence of SV1, SV2, and SV3 was at rates of 40.2%, 0.13%, and 0.04%, respectively. Thus, somaclonal variation rate at the phenotypic level for regenerated cv. Camouflage plants was 40.4% (Table 4-3). Of the 384 regenerated plants of cv. Camille, 10 variants had lanceolate leaves resulting in a somaclonal variation rate of 2.6% (Table 3). No variation was observed among the 62 regenerated plants of cv. Star Bright.
Culture Duration Effects on Somaclonal Variation

Duration of callus culture phase had no effect on somaclonal variation of cv. Camouflage. The somaclonal variation rates for plants regenerated from 8 and 16 months callus culture were 41.2% and 39.5% respectively (Table 4-4).

Effects of Callus Subculture Number on Shoot Regeneration

The 3 Dieffenbachia cultivars displayed significant differences in sustained capacity for shoot regeneration in long-term callus culture (Figure 4-3). Cultivar Camouflage showed a greater capacity for shoot regeneration than cvs. Camille and Star Bright. For cv. Camouflage, the number of shoots per callus increased from 4.7 at the first subculture to 6.3 by the sixth subculture, and then gradually decreased to 2.9 by the twelfth subculture. For cv. Camille, the number of regenerated shoots per callus increased from 2.6 at the first subculture to 4.0 by the fifth subculture, then decreased to 2.8 at seventh subculture, and finally decreasing to no regeneration by the eighth subculture (Figure 4-3). Cultivar Star Bright exhibited no capacity for sustained callus culture as calli lost shoot regeneration ability after the first subculture. Calli of cv. Camille maintained the capacity for regeneration in vitro up to 16 months while cv. Camouflage calli retained regenerative after 24 months culture in vitro.

Discussion

Somaclonal Variation and Genotypes

The present study demonstrated that somaclonal variation occurred among Dieffenbachia plants regenerated through indirect shoot organogenesis from leaf-derived calli. The variants showed distinct phenotypes from their parents. These variants, particularly those isolated from regenerated cv. Camouflage, could potentially be new commercial cultivars as their phenotypes are novel and stable as demonstrated by their progenies after cutting propagation. Additionally, the growth rates of these variants were either higher than or comparative to those of their
parental plants in our shaded greenhouse evaluation. Research thus far has primarily emphasized phenotypic evaluation. This is because in the absence of reliable genetic markers of somaclonal variation and considering the fact that *Dieffenbachia* is primarily propagated through asexual means, phenotype represents the fastest and the most convenient way to identify somaclonal variants. In addition, ornamental plants are prized for their visual phenotypic appearance. As long as a novel phenotype has ornamental value and is stable, it could potentially become a new cultivar.

The rate of somaclonal variation differed greatly, ranging from none for cv. Star Bright and 2.6% for cv. Camille to 40.4% for cv. Camouflage. The low number of regenerated plants, 62 and 384 for cvs. Star Bright and Camille respectively, may be a factor for the low rates of variation observed. It is believed, however, that the genetic makeup of the cultivars plays an important role. First, cvs. Camouflage, Camille, and Star Bright differed in callus formation frequencies, 96%, 62%, and 52% respectively (Table 3-1). Second, the cultivars also varied with respect to *in vitro* shoot formation (Figure 4-3). Calli of cv. Star Bright had no capacity for subculture, and calli of cv. Camille gradually lost the ability to form shoots at approximately 16 months subculture. In contrast, calli of cv. Camouflage maintained their capability to form shoots for at least 24 months after being subcultured. Cultivar differences in the frequency of callus and shoot formation frequencies resulted in low numbers of regenerated plants for cvs. Camille and Star Bright. Thus, it is possible that a prerequisite for producing somaclonal variants through indirect shoot organogenesis is that calli maintain a high and prolonged capacity for shoot organogenesis. Chen et al (2004) reported that cv. Camouflage significantly differed from cvs. Camille and Star Bright genetically as the former was positioned in one genetic cluster while the latter two shared a common cluster. This might partially explain why cv. Camouflage exhibited
greater ability to form calli and shoots in vitro than cvs. Camille and Star Bright. High multiplication rate is associated with rapid mitosis, thus more error may have occurred.

Somaclonal variation rates varying among species and cultivars have been widely documented. Skirvin et al (1994) stated that the somaclonal variation rate expected in vitro was probably about 1% to 3%. Our result with cv. Camille was similar to this rate since 2.6% somaclonal variation occurred in this cultivar. The rate of somaclonal variation in cv. Camouflage was high (40.4%) but not unique. High rates have been reported in other plants as well. Legkobit and Khadeeva (2004) noted 50% to 80% somaclonal variation from Stachys (betony) species. Bairu et al (2006) reported somaclonal variation in Cavendish banana cultivars was high as 72%. The high somaclonal variation rate may also be due to the multiplication of variant cells already produced in the previous cycle since regenerated plants were derived from calli subcultured up to 8 cycles at 8 weeks interval for cv. Camouflage. Xie et al (1995) reported that a particular labile portion of the rice genome was susceptible to stress (in vitro culture condition) and showed higher rearrangement and mutation rates than other portion during in vitro culture. Further research is warranted to determine if the deviant phenotypes resulted from genetic or epigenetic changes and why such a high rate occurred in cv. Camouflage.

Callus Culture Duration

It is generally believed that the rate of somaclonal variation increases with the time a culture has been maintained in vitro. The longer a culture is maintained in vitro, the greater the somaclonal variation (Rodrigues et al., 1998; Kuznetsova et al., 2006). However, the present study showed that for Dieffenbachia cv. Camouflage there was no difference in the rate of somaclonal variation between plants regenerated from 8 and 16 months callus culture. It is not known at this time if shortening the duration of each subculture (less than an 8 week interval)
and increasing time in maintenance or changing growth regulator combinations in media could result in any difference in somaclonal variation rates of cv. Camouflage.

Both the type and the rate of somaclonal variation are crucial factors for determining the feasibility of using somaclonal variation for new cultivar development. In this study, wide variation in leaf variegation, color, shape and plant forms were observed, and these traits are novel and stable demonstrated by their progenies after cutting propagation. These results showed that selection of somaclonal variants has great potential for new cultivar development in *Dieffenbachia*. A future study will focus on analysis of genetic changes of the somaclonal variants using molecular marker techniques.
Figure 4-1. Plants of *Dieffenbachia* cv. Camouflage regenerated by indirect shoot organogenesis showing variation in leaf variegation and color. A) Parental plant: creamy, camouflaged leaves with random green batches of different size. Bar = 10 cm. B) SV1: solid dark green leaves with whitish variegation along the midvein. Bar = 10 cm. C) SV2: light green leaves with many yellowish spots, and connections among spots resulted in large yellowish blotches. Bar = 10 cm. D) SV3: green leaves with few scattered yellowish spots. Bar = 10 cm.
Table 4-1. Quantitative evaluation of somaclonal variants of *Dieffenbachia* cv. Camouflage regenerated by indirect shoot organogenesis and grown in the greenhouse for 8 months.

<table>
<thead>
<tr>
<th>No. of plants</th>
<th>Plant height (cm) ± SE</th>
<th>Canopy height (cm) ± SE</th>
<th>Canopy width (cm) ± SE</th>
<th>Largest leaf length (cm) ± SE</th>
<th>Largest leaf width (cm) ± SE</th>
<th>Basal shoot number ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental type</td>
<td>114</td>
<td>22 ± 0.9 a</td>
<td>39 ± 1.0 a</td>
<td>47 ± 1.0 a</td>
<td>30 ± 0.4 a</td>
<td>11 ± 0.2 a</td>
</tr>
<tr>
<td>SV1</td>
<td>34</td>
<td>30 ± 1.4 b</td>
<td>44 ± 1.6 b</td>
<td>53 ± 1.6 b</td>
<td>33 ± 0.6 b</td>
<td>12 ± 0.3 a</td>
</tr>
<tr>
<td>SV2</td>
<td>3</td>
<td>15 ± 4.7 a</td>
<td>35 ± 5.3 a</td>
<td>41 ± 5.2 a</td>
<td>26 ± 2.0 c</td>
<td>12 ± 1.0 a</td>
</tr>
<tr>
<td>SV3</td>
<td>1</td>
<td>26</td>
<td>17</td>
<td>24</td>
<td>22</td>
<td>8</td>
</tr>
</tbody>
</table>

Means followed by the same letter in each column are not significant at 0.05 level.

Parental type: regenerated plants exhibited the same leaf variegation as the parent.
SV1, SV2, SV3: three types of somaclonal variation observed in cv. Camouflage.
Figure 4-2. Plants of *Dieffenbachia* cv. Camille regenerated by indirect shoot organogenesis showing variation in leaf shape. A) Parental type plants with oblong shaped leaves. Bar = 1 cm. B) Somaclonal variants with lanceolate leaves. Bar = 1 cm.
Table 4-2. Quantitative evaluation of somaclonal variants of *Dieffenbachia* cv. Camille regenerated by indirect shoot organogenesis and grown in the greenhouse for 8 months.

<table>
<thead>
<tr>
<th></th>
<th>No. of plants</th>
<th>Plant height (cm) ± SE</th>
<th>Canopy height (cm) ± SE</th>
<th>Canopy width (cm) ± SE</th>
<th>Largest leaf length (cm) ± SE</th>
<th>Largest leaf width (cm) ± SE</th>
<th>Leaf length/leaf width ± SE</th>
<th>Basal shoot number ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental type</td>
<td>30</td>
<td>6.0 ± 0.2 a</td>
<td>11 ± 0.3 a</td>
<td>10 ± 0.2 a</td>
<td>5.1 ± 0.2 a</td>
<td>3.4 ± 0.1 a</td>
<td>1.5 ± 0.1 a</td>
<td>3.6 ± 0.2 a</td>
</tr>
<tr>
<td>Variant type</td>
<td>10</td>
<td>6.7 ± 0.3 b</td>
<td>13 ± 0.6 b</td>
<td>12 ± 0.4 b</td>
<td>7.0 ± 0.3 b</td>
<td>2.1 ± 0.3 b</td>
<td>3.4 ± 0.1 b</td>
<td>3.8 ± 0.4 a</td>
</tr>
</tbody>
</table>

Means followed by the same letter in each column are not significant at 0.05 level. Parental type: regenerated plants exhibited the same leaf shape as the parent. Variant type: regenerated plants exhibited different leaf shape.
Table 4-3. Effects of genotype on the number and rate of somaclonal variation among *Dieffenbachia* plants regenerated by indirect shoot organogenesis and grown in the greenhouse for 8 months.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>No. of plants regenerated</th>
<th>No. of somaclonal variants</th>
<th>% of somaclonal variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>SV1</td>
</tr>
<tr>
<td>Camouflage</td>
<td>2248</td>
<td>908</td>
<td>904</td>
</tr>
<tr>
<td>Camille</td>
<td>384</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Star Bright</td>
<td>62</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

SV1, SV2, SV3: three types of somaclonal variation observed in cv. Camouflage.
Table 4-4. Effects of the duration of callus culture on the number and rate of somaclonal variation of *Dieffenbachia* cv. Camouflage regenerated by indirect shoot organogenesis and grown in the greenhouse for 8 months.

<table>
<thead>
<tr>
<th>Duration (months)</th>
<th>No. of plants regenerated</th>
<th>No. of variants</th>
<th>Parental type</th>
<th>SV1</th>
<th>SV2</th>
<th>SV3</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1171</td>
<td>483 (41.2)*</td>
<td>688 (58.8)</td>
<td>479 (40.9)</td>
<td>3 (0.26)</td>
<td>1 (0.09)</td>
</tr>
<tr>
<td>16</td>
<td>1077</td>
<td>425 (39.5)</td>
<td>652 (60.5)</td>
<td>425 (39.4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Figures in parenthesis are the rate of somaclonal variation.
Parental type: regenerated plants exhibited the same leaf variegation as the parent.
SV1, SV2, SV3: three types of somaclonal variation observed in cv. Camouflage.
Figure 4-3. Effects of subculture number on shoot regeneration (shoots/callus ± SE) from calli of the 3 *Dieffenbachia* cultivars. Calli were subcultured at 8 week intervals on the callus induction medium under a 16 h light photoperiod. Shoots/callus were recorded after 8 weeks culture on shoot induction medium under a 16 h light photoperiod.
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BIOGRAPHICAL SKETCH

Xiuli Shen was born in Harbin city, Heilongjiang province, China, on October 2, 1964. She grew up in a happy family with loving parents and two younger brothers. From a very young age, she showed her love for science and nature. She was an excellent student and did very well in every subject in school. After graduating from high school in 1982, she moved to Changchun and enrolled at Jinlin University and received a Bachelor of Science degree in July 1987 with a major in molecular biology. Then she moved back to her home city and worked in the Department of Biotechnology in the Northeast Agricultural University as assistant professor from 1987 to 1996, and as an associate professor from 1996 to 2000.

To fulfill her dream to look at the world outside of China and to be able to read a novel written in a foreign language, she came to Saskatoon, Saskatchewan, Canada with her 9-year-old son in May 2000. After 3 years of studying at the University of Saskatchewan, she earned her Master of Science degree in plant science in 2003. She worked as intern in the Plant Biotechnology Institute, National Research Council of Canada during summer 2003. In August 2003, she entered graduate school at the University of Florida to work on a doctoral program in horticultural science. She expects to earn her Ph.D. in May 2007. She has one son, Hao Xu, living with her in Gainesville.