ENDOPOLYPLOIDY IN *DENDROBIUM* CHAO PRAYA SMILE AND *ANTHURIUM ANDRAEANUM* CV 'RED HOT'

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LIST OF ABBREVIATIONS

BA	Benzyl-aminopurine
CDC6	Cell division cycle 6
CYC/CDK	Cyclin/cyclin-dependent kinases
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	Ethylene-diaminetetraacetic acid
GA	Gibberellic acid
IAA	Indole-3-acetic acid
2-iP	6-(γ-γ-dimethylallylamino) purine
KC	Knudson C
KRP	Kip-related protein
MS	Murashige and Skoog
NAA	α-naphthaleneacetic acid
PAC	Paclobutrazol
PCIB	α-(p-chlorophenoxy) isobutryic acid
TIBA	2,3,5-triiodobenzoic acid

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SUMMARY

Endopolyploidy profiles of greenhouse-grown and aseptically-grown *Dendrobium* Chao Praya Smile and *Anthurium andraeanum* cv 'Red Hot' were investigated using flow cytometric analysis. For *D.* Chao Praya Smile, the occurrence of systemic endopolyploidy, with nuclear DNA content ranging from 2C to 32C, was detected in both greenhouse-grown plants and aseptically-grown seedlings. Multiploid cells were found in all the tissues analysed except in seeds where only 2C nuclei were detected. Endoreduplication was observed to be developmentally regulated in the cells of protocorms, leaves and roots, but not in the flowers. In the flowers of *D.* Chao Praya Smile, higher ploidy level was observed in the cells of column as compared to the dorsal and lateral sepals, petals, labellum and pedicel. Similar phenomena were observed in the aseptically-grown seedlings. Protocorms cultured in media containing BA resulted in a decrease in endoreduplication. Conversely, the presence of 2,4-D or GA_3 in the culture medium increased ploidy variation in the protocorms. Addition of TIBA or PAC to the culture medium only inhibited endoreduplication in the protocorms after 6 weeks of culture. On the other hand, the nuclei of greenhouse-grown plants and tissue-cultured plantlets of *A. andraeanum* cv 'Red Hot' were relatively stable with minimal ploidy variations. Only nuclei with 2C and 4C DNA content were detected in the leaves, petioles, roots and spathes. However, nuclei with up to 8C DNA content were detected in the spadices of greenhouse-grown plants. In the callus tissues of *A. andraeanum* cv 'Red Hot', addition of 2,4-D or BA to the culture medium had no effect on endopolyploidy variation. The possible relation between somaclonal variation and endopolyploidy in the explant tissues is discussed.

Chapter 1 Introduction

Endopolyploidy, a result of endoreduplication, has been reported to be common in angiosperms, but not gymnosperms (Barow 2006). Endoreduplication occurs when the normal cell cycle is disrupted and an endonuclear chromosome duplicates in the absence of intervening segregation and cytokinesis (Joubes and Chevalier 2000). The occurrence of endopolyploidy is thought to be family-specific (Barow 2006). For instance, endopolyploidy is common in members of the Cucurbitaceae and Orchidaceae families, but not in members of the Araceae and Liliaceae families (Barow 2006). Endopolyploidy has been observed in different tissues and is spatially and temporally regulated in plants such as *Arabidopsis* (Galbraith et al. 1991), cabbage (Kudo and Kimura 2001a), cucumber (Gilissen et al. 1993), tomato (Smulders et al. 1994), orchids (Lim and Loh 2003, Yang and Loh 2004) and ice plant (De Rocher et al. 1990). The extent of endopolyploidy in plants has been found to be affected by both endogenous (genetic variations and plant growth regulators) and exogenous (light, temperature, nutrients and presence of symbionts or parasites) factors (Barow 2006).

The occurrence of endopolyploidy in plants is suggested to be one of the possible mechanisms of somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variants are considered undesirable if clonal materials are required (Vajrabhaya 1977). Nevertheless, the ability to generate variations in a control manner could be beneficial for crop improvement (Gould 1986). However, somaclonal variation occurs randomly (Larkin and Scowcroft 1981) and the mechanism of somaclonal variation is poorly understood (Puente et al. 2008). One of the possible causes of somaclonal variation could be the pre-existing genetic differences in somatic cells of the initial explants (Evans 1989). Explants that exhibit endopolyploidy would contain a mixture of cells

of varying ploidy levels and shoots with different ploidy levels might be regenerated from these explants (Lim and Loh 2003). Thus culturing explants with multiploid cells might be a cause of somaclonal variation. Therefore, an insight to the endopolyploidy profiles of plants would be useful in the understanding of the nature of explant tissues and provide a possible explanation to the role of endoreduplication in somaclonal variation.

In the present study, the endopolyploidy profiles of two horticulturally important plants, an orchid hybrid (*Dendrobium* Chao Praya Smile) and an anthurium hybrid (*Anthurium andraeanum* cv 'Red Hot') were analysed. The effects of plant growth regulators on endopolyploidy in the protocorm cultures of *D.* Chao Praya Smile and the callus tissues of *A. andraeanum* cv 'Red Hot' were also determined. The objectives of this study are: (1) to analyse the occurrence of multiploid cells in different tissues; (2) to study on the patterns of endopolyploidy throughout the development and (3) to examine the effects of selected plant growth regulators on endopolyploidy in the protocorm cultures of *D.* Chao Praya Smile and callus tissues of *A. andraeanum* cv 'Red Hot'.

Chapter 2 Literature review

2.1 The cell cycle and plant cell cycle

The typical cell cycle is considered as the mechanism for cell growth and development. Stringent control of the cell cycle is required to ensure that the complete genome is only duplicated once per cell cycle, so as to maintain the genome integrity during the development of a multicellular organism (Francis 1998, Gutierrez et al. 2002). Cell cycling occurs in proliferative cells (Francis 1998, Francis 2007) and involves the accurate duplication, segregation of the chromosomal DNA and division of cell leading to the passing of genetic information from one mother cell to two daughter cells (Joubes and Chevalier 2000).

The cell cycle composes of four distinct phases, namely G_1, S, G_2 and M phase (Fig 2.1) (Joubes and Chevalier 2000). At the G_1 phase, which is also known as the post-mitotic interphase, the cell will grow and integrate the relevant signals that will trigger the cell to enter into the S phase and progress through the rest of the cell cycle (Dewitte and Murray 2003). In the S phase, replication of DNA occurs. The DNA content in a nuclear doubles (increases from 2C to 4C, where C is the DNA content of the haploid nuclear genome complement). After the S phase, it proceeds to G_2 phase. At G_2 phase, materials that are needed for nuclear and cell division accumulate and cytoskeletons are reorganised. These allow the separation of chromosome to occur. The cell then proceeds to M phase where mitosis occurs. The whole cell cycle ends when cytokinesis occurs and two daughter cells are formed (Dewitte and Murray 2003). Depending on the environmental conditions, availability of nutrients and plant growth regulators, the newly divided cells would either exit or re-enter into another cell cycle (Doonan 2005).

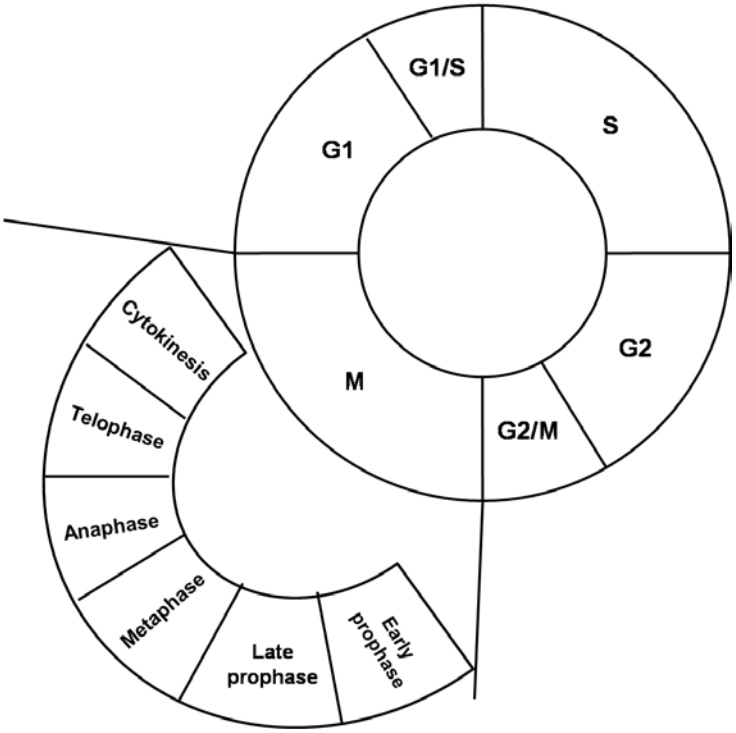

Fig 2.1. The classical cell cycle (adapted from Brooker 1999). See text for detailed description of each stage.

For proliferative cells to complete the whole cell cycle, they must be competent to pass through the checkpoints of cell cycle, namely late G_1 (G_1/S) and late G_2 phase (G_2/M) (Fig 2.1) (Francis and Inze 2001). The competency of the proliferative cells to complete the whole cell cycle is influenced by the availability of nutrients, plant growth regulators and environmental conditions such as light and water (Francis and Inze 2001). If these factors are lacking, the cell cycle will be arrested at the G_1/S or G_2/M transition phase. The G_1/S and G_2/M transition phases are the two main phases where the extracellular signals seem to act on (Doonan 2005). The effects and nature of these signals are tissue dependent and vary with developmental stages (Doonan 2005).

During these phases in the cell cycle, the co-ordinated assembly, activation and sequential inactivation of specific cyclin/cyclin-dependent kinases complexes (CYC/CDK complexes) occur to ensure that the DNA contents in the cells replicate only once per cycle (Fowler et al. 1998). CDKs belong to a class of Ser/Thr kinases and their kinase activities depend on their association with cyclin (CYC) proteins (Churchman et al. 2006). Studies have shown that D-type cyclins (CYCD) regulated the mitotic cycles at the G_1/S transition and over-expression of CYCD3;1 resulted in multicellular trichomes and inhibited endoreduplication in the trichomes of *Arabidopsis* (Schnittger et al. 2002, Dewitte et al. 2003). Hence, genes that regulate cell cycle would influence the endoreduplication cycle in plant cells (Sabelli and Larkins 2007). Over-expression of CDK inhibitors such as Kip-related proteins (KRPs) and SIAMESE (SIM) gene was reported to be able to inhibit cell and endoreduplication cycles (Verkest et al. 2005, Churchman et al. 2006). Hence, the mitotic cell cycle and the endoreduplication cycle might share the same machinery even though the regulatory mechanisms controlling the transition between both cycles have yet to be fully elucidated (Verkest et al. 2005).

The growth and development of eukaryotic organisms depend on the stringent spatial and temporal coordination of cell proliferation, cell differentiation and cell specialisation (Coffman 2004). The overall control of the cell cycle is conserved in all eukaryotic organisms (Murray et al. 2001, De Jager et al. 2005) and orthologs of cell cycle genes have also been found in plants (Sabelli et al. 1996, Springer et al. 2000, Castellano et al. 2001, De Jager et al. 2001, Ramos et al. 2001, Castellano et al. 2004).

However, plants exhibit unique growth characteristics, developmental patterns and body architectures (Kondorosi and Kondorosi 2004). Unlike animal, plant cells that

are involved in cell cycle reside only in the meristem regions (Anova and Rost 1998, Doonan 2005). In plants, formation of adult organs and structures occur after seed germination instead of embryogenesis (Doonan 2005). Many plant cells also have the potential to de-differentiate in response to external signals, such as pathogen infection, wounding, and plant growth regulator treatments (Kondorosi and Kondorosi 2004). Thus the control of the entry and exit from the cell cycle in plant cells is more flexible than that in the animal cells. This flexibility allows the plants which are sessile to better adapt to the environment (Kondorosi and Kondorosi 2004).

Plant meristem consists of a mixture of non-cycling, slowly cycling and rapidly cycling cells (Murray et al. 2001, Doonan 2005). Given the appropriate signals (abiotic or biotic), the meristem cells will continuously divide, to form a new layer of meristem cells, while the older cells behind the new meristem cells will differentiate to produce new organs such as leaves, roots and flowers (De Jager et al. 2005, Doonan 2005). It has been proposed that differentiating cells will exit cell cycle and become quiescent or enter endoreduplication cycle (Kondorosi and Kondorosi 2004). Endoreduplication cycle is believed to be the switch between cell proliferation and cell differentiation during the developmental stages (Jasinski et al. 2002). This cycle shares several characteristics with the mitotic cycle and is considered to be a modified form of the typical cell cycle (Joubes and Chevalier 2000). Studies on endoreduplication and cell cycles are often carried out with flow cytometer (Yanpaisan et al. 1999).

2.2 Flow cytometry as a tool to investigate endopolyploidy in plants

Flow cytometry is a technique used for measuring or analysing the property of single biological particles such as cells, nuclei and organelles in a fluid suspension (Dolezel et al. 1994, Yanpaisan et al. 1999, Carter and Ormerod 2000). These cells are usually

stained by fluorochromes. The intensity of fluorescence given out by each cell will be measured by the flow cytometer. The results can be shown as peaks in the form of histograms (Yanpaisan et al. 1999).

A flow cytometer basically consists of a fluidics system, an optical system and a signal processing system (Carter and Ormerod 2000). The fluidics system will deliver the suspended particles from the sample individually into the sensing region of the flow chamber where the light is focused. The light source is usually an argon-ion gas laser, which emits light at a specific wavelength to excite the fluorochromes (Carter and Ormerod 2000). The emitted fluorescence will be detected and recorded by the photomultiplier tubes and photodiode of the optical system. The light signals will subsequently be converted to proportional electronic signals that are digitised for computational analysis (Carter and Ormerod 2000). For the determination of absolute DNA amount, the fluorescence intensities of nuclei of internal or external standard and sample population are compared (Arumuganathan and Earle 1991). Background or noise due to debris with low fluorescence can be eliminated from the analysis by creating a gating region around the signals due to intact nuclei on a bivariate histogram (Arumuganathan and Earle 1991). For each sample, about 10 000 nuclei are usually analysed as large sample size improves the accuracy of the reading (Galbraith 1990). In addition, the accuracy of the reading is also influenced by factors intrinsic to the instrument and factors associated with sample preparation and material used (Galbraith 1990). Natural pigments (chlorophyll) and organelles that auto-fluoresce would reduce the resolution of the analysis (Bergounioux and Brown 1990, Galbraith and Lambert 1996). Furthermore, certain flurochrome stains which bind preferentially to the AT-rich or the GC-rich regions of the DNA would result in inaccuracy when determining the absolute amount of nuclear DNA from AT-rich or GC-rich samples

(Dolezel and Bartos 2005). Therefore, the selection of an appropriate flurochrome stain and optimisation of instrumentation settings are essential when using a flow cytometer.

Before the application of flow cytometry in plant system, Feulgen microdensitometry and microspectrofluorometry were the main methods used in determining ploidy levels and DNA contents in the plants. In comparison to these cytophotometric methods, flow cytometry is a more rapid, convenient and sensitive technique for analysing large nuclei population (Arumuganathan and Earle 1991, Dolezel 1991, Jones et al. 1998). Most of the flow cytometry methods have been developed using animal systems (Yanpaisan et al. 1999). Plant cells are usually much larger than the animal cells (mammalian blood and lymphoid cells) for which the flow cytometer was initially designed for (Galbraith 1989). Therefore, modifications to the flow cytometer are required before it can be used in plant research (Galbraith et al. 1983). The first attempt to use flow cytometry in plants was done by Heller (1973). However, application of this technique in plant research was limited by the lack of investment in plant cell biology as compared to animals and problems in the preparation of intact plant cells and nuclei suspension that were suitable for flow cytometry (Dolezel and Bartos 2005). This technique was only adapted and widely used for the application to plant cells after Galbraith et al. (1983) reported a simplified and rapid isolation protocol. Modifications to this protocol allow rapid analysis of DNA and RNA contents, karyotyping, cell counting, studying of chloroplasts and selection of particular cells or subcellular organelles of interest (Yanpaisan et al. 1999). Flow cytometry is a powerful tool for fast and accurate detection of DNA contents and endopolyploidy in both animal and plant systems (Arumuganathan and Earle 1991, Biradar and Rayburn 1993, Yanpaisan et al. 1999). It has been used for the analyses of

endoreduplication of a variety of tissue types and development stages of *Arabidopsis* (Galbraith et al. 1991), cabbage (Kudo and Kimura 2001a), orchids (Lim and Loh 2003, Yang and Loh 2004) and ice plant (De Rocher et al. 1990).

Flow cytometry and cell sorting require the sample to be a single intact cell or nuclei suspension (Galbraith 1989). Therefore, the accuracy of the analysis depends on the quality of the cells or nuclei suspension. As higher plants comprise of a complex three-dimensional structure of inter-connected tissues with cells having thick cellulose walls, nuclei isolation is more difficult than animals (Bergounioux and Brown 1990). During the preparation of nuclei suspension, sample preparation methods and the composition and pH of the extraction buffer are critical in ensuring the quality and quantity of the nuclei (Dolezel and Bartos 2005). Intact nuclei can be isolated from the plant cells and tissues by direct chopping with a razor blade in the extraction buffer (Galbraith et al. 1983, Lim and Loh 2003), crushing with a glass rod in buffer (Lim and Loh 2003), beating with beads in buffer (Roberts 2007) or grinding in a small homogeniser (Rayburn et al. 1989). Among them, the direct chopping method is the most commonly used (Yanpaisan et al. 1999). However, extensive chopping, which causes nuclear damage and generates more debris, has to be avoided (Dolezel et al. 1994). Modifications such as freezing plant suspensions before chopping and fixing plant materials before or after isolation are made to improve the quantity and quality of nuclei suspension (Yanpaisan et al. 1999).

As the stability of plant nuclei declines with time, the isolation of nuclei must be carried out on ice and analyses with flow cytometer must be carried out within 24 hours (Galbraith 1989, Arumuganathan and Earle 1991, Dolezel et al. 1994). Furthermore, during the isolation of nuclei, proper ratio of plant material to extraction

buffer must be used (Arumuganathan and Earle 1991). A high plant material to extraction buffer ratio will result in an increase in the amount of cellular debris and interfere with analysis, while a low plant material to extraction buffer ratio will dilute the nuclei concentration in the sample. In *Dendrobium*, it was found that young leaf tissues would produce mucous exudates that interfere with nuclei extraction (Jones and Kuehnle 1998). In such case, a low plant material to extraction buffer ratio is preferred.

The functions of the extraction buffer are to ensure the release of nuclei, maintain the integrity of the nuclei by protecting the nuclei DNA against endonucleases and facilitate DNA staining (Dolezel and Bartos 2005). Due to the diversity in tissue anatomy and chemistry among plant species, no single extraction buffer is universally optimum for all plants (Dolezel and Bartos 2005). Modifications are made to the existing extraction buffers to obtain one that is optimal for the plant material used. Magnesium ions and spermine are usually used in the buffers to stabilise the nuclear chromatin. Metal chelator such as ethylene-diaminetetraacetic acid (EDTA) is used to bind the divalent cations which are cofactors of nucleases (Dolezel and Bartos 2005). Furthermore, glucose is sometimes added to help in maintaining nuclear integrity and preventing the clumping of nuclei (Dolezel and Bartos 2005). Inorganic salts such as KCl and NaCl are added to achieve the adequate ionic strength, while surfactants such as Triton X-100 and Tween 20 are included to facilitate the release of nuclei from the cytoplasm, remove cytoplasmic remnants from the surface of isolated nuclei, disperse chloroplasts and prevent the aggregation of nuclei with the cytoplasmic debris (Dolezel and Bartos 2005). To improve the cell cycle resolution and nuclear extraction of certain plant material, 0.5 to 1.0 % (v/v) of Triton X-100 is used (De La Pena and Brown 2001). When browning (due to release of phenolic compounds) occurs,

reducing agents (dithiothreitol or β-mercaptoethanol) or protectants (polyvinyl pyrrolidone) are used to preserve chromatic proteins and counteract the interference of phenolic compounds with DNA staining (De La Pena and Brown 2001). Organic buffers such as Tris, MOPS and HEPES are often used to stabilise the pH of the buffer at the range of 7.0 to 8.0, which is compatible with the common DNA fluorochromes (Dolezel 1991, Dolezel and Bartos 2005).

The DNA fluorochromes that are commonly used in flow cytometry are propidium iodide, 4,6-diamidino-2-phenylindole (DAPI), Hoechst dyes and mithramycin (Dolezel 1991, Dolezel and Bartos 2005). Propidium iodide is a DNA intercalator and binds to double-stranded DNA and RNA. Therefore, samples that are stained with propidium iodide have to be pre-treated with RNase (Yanpaisan et al. 1999, Dolezel and Bartos 2005). Hoechst dyes and DAPI are easy to excite and measure by flow cytometer. They bind to double-stranded DNA and their bindings are not influenced by chromatin structure which would reduce the resolution of the peaks. They bind preferentially to AT-rich region, while mithramycin is specific to GC-rich region (Yanpaisan et al. 1999, Dolezel and Bartos 2005). Therefore, inaccuracy will be resulted if these stains are used for the analyses of AT-rich or GC-rich samples. Thus the choice of fluorochromes used is dependent on the resolution, stability of the fluorochromes, incubation time, excitation wavelength available in the flow cytometer, compatibility with other simultaneous staining, DNA stiochiometry and cost (Yanpaisan et al. 1999).

2.3 Endoreduplication in plants

Cell polyploidisation, also known as endoployploidisation, has been reported to be a widespread occurrence in eukaryotes (Brodsky and Uryvaeva 1977). Endopolyploidy

is generally used to describe the result of multiple doubling (2^n) of nuclear DNA without the occurrence of nuclear division (Joubes and Chevalier 2000). In both animal and plant, endopolyploidy in the somatic cells is mainly due to either endomitosis or endoreduplication (D'Amato 1964, Brodsky and Uryvaeva 1977, Joubes and Chevalier 2000). Geitler (1939) first reported on the occurrence of endomitosis (Joubes and Chevalier 2000). Unlike the classical cell cycle, endomitosis occurs in the absence of mitotic spindle and cytokinesis. After each round of endomitosis, chromosome number in the cells doubles (Fig 2.2) (Joubes and Chevalier 2000). Its occurrence is reported in several animal groups and rarely in the angiosperms (D'Amato 1984).

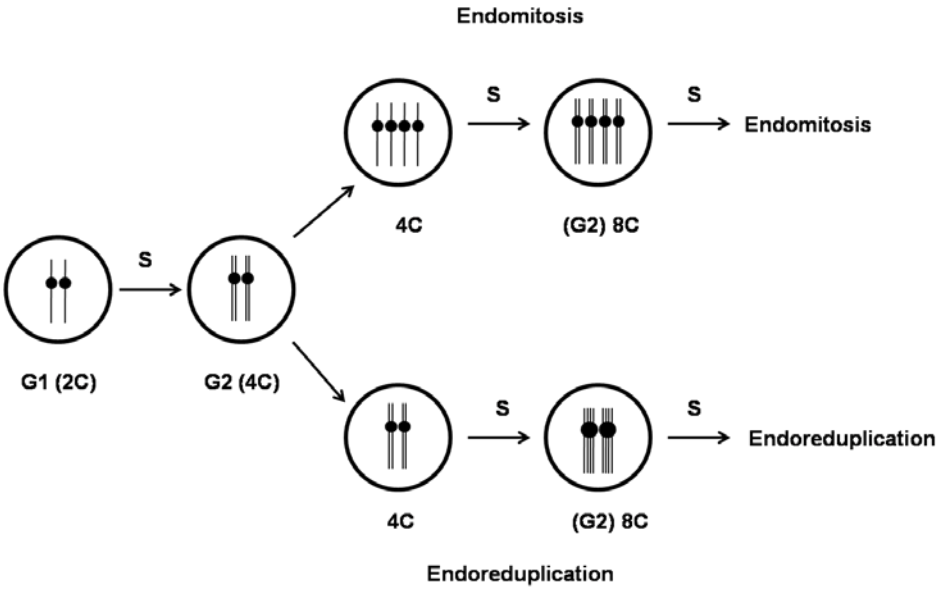

Fig 2.2. Comparison of endomitosis and endoreduplication (One pair of chromosome is shown) (adapted from D'Amato 1984).

On the other hand, endoreduplication has been reported to be common in many plant species (Joubes and Chevalier 2000, Barow 2006). It has been observed in over 90 % of angiosperms (D'Amato 1984), but its occurrence is not common in gymnosperms (Barow 2006). It was first reported by Levan (1939) to occur in the elongation zone of onion roots that were subjected to auxin treatment. Unlike endomitosis, endoreduplication does not result in an increase in chromosome number in each nuclear. Instead it leads to the production of chromosomes with 2^n chromatids (Fig 2.2) (Lorz 1947, Levan and Hauschka 1953). The degree of endopolyploidy might differ with nuclei, thus resulting in a tissue possessing a mixture of cells of varying ploidy levels that are a multiple of 2C (Joubes and Chevalier 2000, Edgar and Orr-Weaver 2001). Endoreduplication has been hypothesised to be an evolutionary alternative for plants with small genome to achieve high nuclear DNA contents so as to support the differentiation and specialised function of certain cells (Nagl 1976, Galbraith et al. 1991). However, such phenomenon was also observed in plants with large genome (Joubes and Chevalier 2000).

Nevertheless, endoreduplication was reported to be common in tissues with specific function and cells of large size (Alvarez 1968, Joubes and Chevalier 2000, Kondorosi et al. 2000, Lim and Loh 2003). It was observed in endosperms of *Zea may* (Schweizer et al. 1995), suspensor cells of *Phaseolus* (Brodsky and Uryvaeva 1977), trichomes of *Arabidopsis* (Melaragno et al. 1993), raphide crystal idioblasts of *Vanilla* (Kausch and Horner 1984), parenchyma of orchid protocorms (Alvarez 1968), root hairs of *Elodea canadensis* (Dosier and Riopel 1978) and basal cells of the hairs of *Bryonia* anthers (Barlow 1975). It has also been observed in other types of tissue such as cotyledons (Dhillon and Miksche 1982) and leaf epidermal cells (Kinoshita et al. 1991, Melaragno et al. 1993).

Its occurrence may bring about certain advantages to the plants to help them better adapt to their environment (John and Qi 2008). Endoreduplication has been shown to be needed for the development of the enlarged symbiotic nodule cells in *Medicago truncatula* and *M. sativa* (Cebolla et al. 1999). Root nodule cells that lacked endoreduplication could not mature into nitrogen-fixing cells and symbiotic bacteria could not enter diploid cells (Vinardell et al. 2003). Endoreduplication during the development of these cells was to ensure that the symbiotic cells were large enough to host the nitrogen-fixing bacteria (Vinardell et al. 2003). It was also to provide the energy and nutrient for the bacteria by increasing transcriptional and metabolic activities of the host cells (Vinardell et al. 2003).

Endoreduplication might also participate in the formation of plant defense mechanisms. Calcium oxalate crystals are one of the defense mechanisms of plants against herbivores (Franceschi and Nakata 2005). These crystals accumulate in the idioblasts (Foster 1956). Since endoreduplication was reported in the idioblasts of *Vanilla planifolia* (Kausch and Horner 1984), this process might be required for the accumulation of calcium oxalate in idioblasts. Another defense mechanism of the plants is the formation of hair-like structures such as trichomes. Trichomes help to reduce the heat load of plants, increase freeze tolerance and protect the plant from ultraviolet light. They also protect the plants from biotic factors such as insects, herbivores and pathogens (Johnson 1975, Mauricio and Rausher 1997, Werker 2000, Serna and Martin 2006). Studies have shown that four rounds of endoreduplication cycle occur in the trichomes of *Arabidopsis* which are unicellular (Hulskamp et al. 1999) and the cell size and degree of branching of trichomes are affected by endoreduplication (Cebolla et al. 1999). Further advantage of endopolyploidy is to aid in the development of endosperm (Leiva-Neto et al. 2004) and tapetal tissues (Weiss

and Maluszynska 2001), which help in the nutrition of the embryos and pollen grains. For instance, during the development of maize endosperm, endoreduplication was required to drive the production of storage proteins and starch which act as nutrient sources for the developing embryo (Lur and Setter 1993).

It has been shown that endopolyploidy in the plant tissues are developmentally regulated. For instance, endopolyploidy in different tissues and its changes throughout development stages have been observed in *Arabidopsis* (Galbraith et al. 1991), cabbage (Kudo and Kimura 2001a), cucumber (Gilissen et al. 1993), tomato (Smulders et al. 1994), orchids (Lim and Loh 2003, Yang and Loh 2004) and ice plant (De Rocher et al. 1990). These studies showed that endopolyploidy in the tissues was spatially and temporally regulated. In most plant species, the percentage of multiploid cells increases as the tissue aged (Joubes and Chevalier 2000, Barow 2006). Furthermore, there are increasing evidences showing a positive correlation between cell size and variation in endopolyploidy (Melaragno et al. 1993, Folkers et al. 1997, Cebolla et al. 1999, Kondorosi et al. 2000). It is speculated that endoreduplication is required for the expansion and differentiation of plant cells which is essential for the specific function of a given type of cell (Kondorosi et al. 2000, Barow 2006). It has also been suggested to be involved in the vegetative growth of plants (De Veylder et al. 2001).

However, cell elongation could be uncoupled from endoreduplication (Gendreau et al. 1998). It was observed that the root cells from different ecotypes of *Arabidopsis* had varied size, but no correlation was found between their cell size and ploidy level (Beemster et al. 2002). Therefore, the involvement of endoreduplication in the vegetative growth of plants has been questioned (John and Qi 2008). It has been

suggested that in the vegetative tissues, the potential to resume cell division is preserved by the scattered distribution of endoreduplicated cells intercalated among surrounding unreduplicated cells which can divide for wound repair (John and Qi 2008). Therefore, endoreduplication is not directly involved during the vegetative growth of the plants as endoreduplication is generally an irreversible process and further cell proliferation is prevented (John and Qi 2008). However, endoreduplicated cells may still have the potential to re-enter normal cell cycle. In the epidermal cells of tobacco hornworms (*Manduca sexta*), cells that had endoreduplicated to 32C would re-enter mitosis and reduced their ploidy back to 2C in an increase in the steroid hormone, ecdysone (Kato et al. 1987). Furthermore, Weinl et al. (2005) reported that *Arabidopsis* cells, which were induced to endoreduplicate by the mis-expression of ICK1/KRP1, could re-enter normal cell cycle.

Besides cell cycle and endoreduplication, other mechanisms might be present in the regulation of the vegetative growth of plants (Sugimoto-shirasu and Roberts 2003). Plant organ growth is also determined by cell number and size (Horiguchi et al. 2006). It has been shown that plants could detect and control the size of an organ and regulate their growth accordingly (Tsuge et al. 1996, Day and Lawrence 2000). Therefore, the growth of cell is influenced by their interactions with the neighboring cells and controlled by other regulatory systems. The coordination of these regulatory networks would lead to the formation of organs (Kondorosi et al. 2000). All these networks might have been interlinked with the cell and endoreduplication cycles. The regulatory mechanisms in different tissues might also differ (Churchman et al. 2006). Hence, it is difficult to establish a general relationship between endoreduplication, cell growth and differentiation during plant development.

2.4 Molecular mechanisms of plant endoreduplication cycle

Many recent studies have been carried out to establish a relationship between cell differentiation and endoreduplication cycle. Regulatory mechanisms have also been proposed (Cebolla et al. 1999, Vinardell et al. 2003, Churchman et al. 2006, Yoshizumi et al. 2006). However, this knowledge is insufficient to fully elucidate the link between development and the degree of endoreduplication in various plant tissues (Churchman et al. 2006, Dewitte et al. 2007). The occurrence of large number of genes encoding the core cell cycle factors in plants and the redundancy in some of the gene functions further complicate genetic analyses (Menges et al. 2005, Dewitte et al. 2007). This redundancy in gene functions might be an adaption to ensure the loss of one component in the regulatory mechanisms could be compensated by another. Recent studies seem to suggest that endoreduplication is spatially and temporally regulated by more than one pathway, which is dependent on the biotic and abiotic conditions that the plants are subjected to (Yoshizumi et al. 2006).

In the normal cell cycle, cells have a mechanism to ensure that chromosomes are replicated only once per cycle (Sugimoto-shirasu and Roberts 2003). However, in endoreduplication cycle, chromosomes in the cells are re-replicated in the absence of mitosis. In the switch from normal cell cycle to endoreduplication cycle, the cells must be able to start another round of DNA replication (S phase), while inhibiting mitosis at the same time (Sugimoto-shirasu and Roberts 2003). For an endoreduplication cycle to re-enter S phase, mechanisms similar to the normal cell cycle could be involved (Sugimoto-shirasu and Roberts 2003, Kondorosi and Kondorosi 2004). Therefore, it has been proposed that the endoreduplication cycle is regulated at the level of replication origin activity, the cyclin-dependent kinase activity, the

retinoblastoma/E2F pathway, and also the degradation of G1/S and G2/M-phase specific factors (Sabelli and Larkins 2007).

The control and regulation at the replication origin has been suggested to be one of the key mechanisms in the switch from cell cycle to endoreduplication cycle (Sabelli and Larkins 2007). Before DNA replication, pre-replication complex (pre-RC) will be assembled at the replication origin (Sabelli and Larkins 2007). The pre-RC is formed by the assembly of origin recognition complex, cell division cycle 6 (CDC6), CDT1 (DNA replication factor) and minichromosome maintenance proteins (Sabelli and Larkins 2007). However, reports on the regulation of pre-RC components and DNA replication licensing in plants are limited (Castellano et al. 2004). In *Arabidopsis*, both AtCDT1 and AtCDC6 exhibited a positive role in the regulation of endoreduplication in the leaf epidermis cells (Castellano et al. 2001, Castellano et al. 2004). Ectopic expression of AtCDC6 induced endoreduplication in leaves and the stability of CDC6 protein was enhanced in cells undergoing endoreduplication (Castellano et al. 2001). Castellano et al. (2004) also showed that in cells that were competent to divide or with limited stem cell potential, an increase in CDT1 and CDC6 levels would result in cell proliferation, while in cells programmed to undergo endoreduplication, extra rounds of endoreduplication cycle would be triggered.

The retinoblastoma-adenovirus E2-promoter binding factor (Rb-E2F) pathway is another proposed mechanism in the regulation of endoreduplication (De Veylder et al. 2002, Shen 2002, Boudolf et al. 2004, Del Pozo et al. 2006). E2F genes are found in *Arabidopsis* (Magyar et al. 2000), carrot (Albani et al. 2000), tobacco (Sekine et al. 1999) and wheat (Ramirez-Parra et al. 1999). De Veylder et al. (2002) reported that ectopic expression of E2Fa-DPa could sustain cell division in cells that were

competent to divide and induce endopolyploidy in endoreduplicating cells. Therefore, the ectopic expression of E2Fa-DPa was proposed to stimulate cell cycle progression by triggering S phase entry and cells with mitosis inducing factor would proceed into mitosis (De Veylder et al. 2002). In cells lacking this mitosis inducing factor, E2Fa-DPa would stimulate S phase re-entry, resulting in the increase in ploidy level (De Veylder et al. 2002). Boudolf et al. (2004) suggested that CDKB1;1 might be part of this mitosis inducing factor. E2Fc/DPb was also reported to be a key component in controlling the switch to endoreduplication cycle. E2Fc was suggested to repress the expression of cell cycle genes and over-expression of E2Fc induced endoreduplication in the cells of *Arabidopsis* (Del Pozo et al. 2006).

Cyclin/CDKs complexes are also reported to be the key components of the regulatory mechanisms of both cell and endoreduplication cycles (Sabelli and Larkins 2007). For instance, over-expression of D-type cyclin (CYCD) in the *Arabidopsis* cells inhibited endoreduplication (Dewitte et al. 2003). Moreover, the loss of CYCD3 function in leaf development would lead to an early onset of endoreduplication (Dewitte et al. 2007). In *Arabidopsis*, the expression of CYCD3;1 gene was reported to be induced by cytokinin, suggesting that the CYCD3 gene family might be a key component in integrating both cell and endoreduplication cycles of plants in response to hormonal signals (Dewitte et al. 2007).

Regulation of endoreduplication also involves the sustaining or up-regulation of S phase CDKs and down-regulation of M phase CDKs. This depends on the time and specific interaction of CDKs with cyclins, which involves processes such as cyclins synthesis, degradation and compartmentalisation (Sauer et al. 1995, Edgar and Orr-Weaver 2001, Larkins et al. 2001, Sabelli and Larkins 2007). It is proposed that the

transition to endoreduplication is promoted by a decrease in the activities of mitotic kinases such as CDKs (Dewitte et al. 2007). These kinases activities can be regulated by anaphase promoting complex (APC) (Vinardell et al. 2003) and CDK inhibitory proteins such as Kip-related protein2 (KRP2) (Schnittger et al. 2003, Verkest et al. 2005), SIM (Walker et al. 2000, Churchman et al. 2006), and NtKIS1a (Jasinski et al. 2002).

The APC is an E3 ubiquitin ligase complex involves in the degradation of key cell cycle proteins. It has been observed to play an important role in the regulation of cell cycle transition such as mitosis exit and DNA replication (Sabelli and Larkins 2007). The ccs52A gene is a plant ortholog of yeast and animal cdh1/srw1/fzr genes. It is a substrate-specific activator of the APC ubiquitin ligase (Cebolla et al. 1999, Vinardell et al. 2003). CCS52A protein is involved in the transition of mitotic to endoreduplication cycle and plays a key role in the formation of large highly multiploid symbiotic cells of the nitrogen-fixing root nodules (Vinardell et al. 2003).

KRP2 levels are more abundant in endoreduplicating than mitotically dividing tissues (Verkest et al. 2005). It inhibits the activity of CDKA;1/cyclin complex during the onset of endoreduplication. Hence, KRP2 might be an activator of the mitosis-to-endoreduplication transition (Verkest et al. 2005). SIM protein is another CDK inhibitor found in *Arabidopsis* and is associated with CYCD and CDKA;1. Over-expression of SIM resulted in an increase in endoreduplication, although this was tissue specific (Churchman et al. 2006).

2.5 Factors affecting endoreduplication in plants

Endogenous (genetic variations and plant growth regulators) and exogenous (light, temperature, water and presence of symbionts or parasites) factors are suggested to affect signals that will initiate endoreduplication and influence the ploidy variation in plants (Barow 2006).

Genetic variations between individuals of the same species belonging to different ecotypes or varieties may result in variations in endopolyploidisation (Barow 2006). It has been shown that there is a negative correlation between ploidy level of plants and endoreduplication. For instance, the seedlings of polyploid sugar-beet (Sliwinska and Lukaszewska 2005), tetraploids of *Portulaca grandiflora* that were obtained by colchicines treatment (Mishiba and Mii 2000) and mesocotyls of tetraploid *Zea mays* (Biradar et al. 1993) had a lower extent of endoreduplication than their corresponding diploids. Moreover, the crossing of two varieties of *Zea mays* that exhibited different endoreduplication patterns in their leaf epidermis resulted in a F1 generation having ploidy levels of the epidermis cells that were intermediate to that of the parents (Cavallini et al. 1997). Contrary to this, no difference in the patterns of endopolyploidy of diploid and tetraploid of tomato (*Lycopersicon esculentum cv.* Moneymaker) has been found (Smulders et al. 1994). Thus the influence of genetic variation on endopolyploidy might be genetically dependent and vary between ecotypes or varieties.

Plant growth regulators such as cytokinins and auxins have been reported to affect cell (Dewitte and Murray 2003, Menges et al. 2006) and endoreduplication cycles (Kende and Zeevaart 1997) in plants. Molecular analysis has shown that cytokinin regulates cell division in the developing leaves and shoot meristem of *Arabidopsis* by inducing

the expression of the cyclin, CYCD3 (Dewitte et al. 2007). CYCD3 is proposed to delay the onset of endoreduplication by extending the "mitotic window" of leaf development (Dewitte et al. 2007). In suspension cultures of *Doritaenopsis*, the presence of benzyl-aminopurine (BA) or thidiazuron in the medium resulted in a decrease in endoreduplication in the cells (Mishiba et al. 2001). However, increased endoreduplication was observed in the mesophyll and abaxial epidermal cells of bean plants (*Phaseolus vulgaris*) watered with BA solution (Kinoshita et al. 1991). In cell cultures of tobacco, the addition of both cytokinin and auxin to the culture medium induced cell division, resulting in DNA deduplication in the cells, while in medium containing auxin, cell elongation and endoreduplication were induced (Valente et al. 1998). In cultured pea root cortex cells, medium containing both auxin and cytokinin induced endoreduplication, but no effect on endopolyploidy was observed in auxin-only medium (Libbenga and Torrey 1973).

Enhancing effect of endoreduplication by exogenous auxin was first observed in the roots of onion after watering with auxin solution (Levan 1939). Increased endopolyploidy was also reported in the fruits of apricot trees that were sprayed with 2,4-dichlorophenoxyacetic acid (2,4-D) solution (Bradley and Crane 1955) and endosperm of maize (*Zea mays*) after the application of lanolin paste containing 2,4-D on the exposed pericarp surface of kernel (Lur and Setter 1993). However, the enhancing effect of auxin varied among plants. An increase in endoreduplication has been observed in the protocorms of *Vanda* Miss Joaquim (Lim and Loh 2003) cultured in medium containing auxin, but auxin has no effect on endopolyploidy in the regenerants of cactus *Mammillaria san-angelensis* cultures (Palomino et al. 1999). In the cells of suspension cultures of *Doritaenopsis*, an increase in endoreduplication was only detected in medium supplemented with 2,4-D or picloram and not other auxins

(Mishiba et al. 2001). Thus different types of auxin have different effect on endoreduplication.

Gibberellic acid (GA) has been suggested to regulate the size of mesocarp cells of tomato by increasing ploidy level in the cells (Serrani et al. 2007). However, the effect of GA on endopolyploidy is variable. For instance, it was found to enhance endoreduplication in one variety of *Pisum sativum*, but had no effect on another (Callebaut et al. 1982). Moreover, the presence of GA_3 only resulted in an increase in endopolyploidy in the leaf cells of *Triticum durum* cultivated in the dark, while another cultivar, Creso, was insensitive to GA_3 in dark and light treatments (Cavallini et al. 1995). The difference in the effect of GA was due to the *Rht 1* gene in *T. durum* cv Creso. It influenced both plant height and sensitivity to endogenous GA (Cavallini et al. 1995). This further supports the hypothesis on the influence of genetic variation on endoreduplication (Barow 2006). Furthermore, in the cells of *V.* Miss Joaquim protocorms, the addition of GA_3 to the culture medium only resulted in a slight increase in endoreduplication (Lim and Loh 2003). In the cells of dark-grown GA-deficient mutants of *Arabidopsis thaliana*, medium containing GA increased ploidy variation (Gendreau et al. 1999). Hence, the effect of GA may be cultivar dependent and vary in different species.

Ethylene regulates a wide variety of developmental processes in plants (Dan et al. 2003). It was reported that ethylene enhanced endoreduplication in the hypocotyl epidermis of cucumber seedlings cultured in container filled with ethylene gas. When ethylene was removed, cytokinesis of cells was observed (Dan et al. 2003). Extra rounds of endoreduplication were also induced in the hypocotyls of light- and dark-grown *Arabidopsis* seedlings after culturing in medium containing 10.0 µM ethylene

precursor (1-aminocyclopropane-1-carboxylic acid) (Gendreau et al. 1999). Therefore, further investigations on the effects of different plant growth regulators on endoreduplication would enhance the understanding of their role in this process.

Plant growth conditions such as light and temperature have also been suggested to influence endopolyploidy (Joubes and Chevalier 2000, Jovtchev et al. 2007). Light is an important environmental factor that regulates plant growth and development throughout its life cycle (Neff et al. 2000, Franklin and Whitelam 2004). The ploidy levels of dark-grown seedlings of *T. durum* (Cavallini et al.1995), *P. sativum* (Van Oostveldt and Van Parijs 1975, Callebaut et al. 1982), *Glycine max* (Galli 1988) and *Arabidopsis* (Gendreau et al. 1998, Tsumoto et al. 2006) were found to be higher than those grown under the light. Furthermore, endoreduplication in *Arabidopsis* has been reported to be regulated by phytochrome which was a photoreceptor that the plants used to detect light (Gendreau et al. 1998). Therefore, endoreduplication might be a mechanism to enhance the elongation of the hypocotyls of dark-grown seedlings in the process of detecting light source.

Alteration in temperature has an influence on the development of plants (Franklin and Whitelam 2004). It could also affect endoreduplication in plants. In chill-sensitive plant such as soybean, chilling reduced its growth and inhibition in endoreduplication was observed in the root cortex and root hairs cells (Stepinski 2003). In orchids, a decrease in growth and endoreduplication transition rates during flower development was observed when the temperature was lowered from 25 to 15 ºC (Lee et al. 2007). A significant decrease in endopolyploidy was also observed in the mesocotyls of maize when the growing mesocotyls were exposed to a temperature change from 23 to 15 ºC for 5 days (Wilhelm et al. 1995). Furthermore, exposure to high temperature (35 ºC

instead of 25 ºC) for 4 to 6 days resulted in a significant decrease in endoreduplication in the maize endosperm and affected its development (Engelen-Eigles et al. 2000).

Due to their sessile lifestyle, plants must be able to adjust their growth to the environmental conditions. Other than the above mentioned, salt stress and water deficit are the common problems that are experienced by the plants. It was found that endoreduplication was induced during the differentiation of root cortex cells of *Sorghum bicolor* watered with increasing concentrations of NaCl and $CaCl_2$ solution (Ceccarelli et al. 2006). Hence, endoreduplication is suggested to be a factor of salt adaptation in *S. bicolor* (Ceccarelli et al. 2006). In the endosperm of maize, the rate of cell division decreased drastically, while cells undergoing endoreduplication increased steadily at the onset of water deficit (Artlip et al. 1995, Setter and Flannigan 2001). However, in the advanced stage of water deficit, endoreduplication and associated S phase processes in the endosperm were both inhibited (Artlip et al. 1995, Setter and Flannigan 2001). Therefore, in the maize endosperm, mitosis appeared to be more sensitive to water stress as compared to endoreduplication (Artlip et al. 1995, Setter and Flannigan 2001). Moreover, study has also shown that polyploid plants were more tolerance to water stress than their diploid counterparts (Li et al. 1996). Thus water and salt stress might affect endopolyploidy variation in plants.

Another factor that has been reported to affect the occurrence of endopolyploidy is the presence of symbionts or parasites. Cells of crown galls have higher degree of ploidy variation than the tissues that they are derived from (D'Amato 1964). The root nodule cells of *P. sativum* (Barow 2006) and *M. truncatula* (Vinardell et al. 2003) that have symbiotic bacteria also exhibited higher degree of endopolyploidy as compared to other root cells. Furthermore, infection of root cells of tomato plants by *Arbuscular*

mycorrhizal had resulted in a significant increase in the degree of endopolyploidy as compared to non-infected cells (Berta et al. 2000). The symbionts or parasites might have produced compounds to induce endoreduplication in the host cells (Schultze and Kondorosi 1998). Endoreduplication in the host cells might be to ensure that the cells are large enough to host the bacteroids and provide sufficient energy and nutrients for the bacteriods (Vinardell et al. 2003).

2.6 Somaclonal variation and endopolyploidy

During plant propagation, maintaining the genetic integrity with respect to the parent plants is very important (Lee and Phillips 1988). However, during micro-propagation of plants, somaclonal variation is common (Lee and Phillips 1988). Somaclonal variation is referred to as the variation arising in the cell cultures, regenerated plants and their progenies during micro-propagation (Brar and Jain 1998). It appears to be non-species specific as variations among tissue-cultured plantlets have been observed in different plants (Larkin and Scowcroft 1981). Variations have been observed in cultures of sugar cane (Singh et al. 2007), potato (Shepard et al. 1980), rice (Nishi et al. 1968), barley (Li et al. 2001), maize (Brettell et al. 1980), rye (Linacero and Vazquez 1993), *Pelargonuium* species (Skirvin and Janick 1976), orchid (Ferreira et al. 2006a) and other plants (Larkin and Scowcroft 1981). Flow cytometry, chromosomal number counter, random amplified polymorphic DNA and simple sequence repeat have been used to detect somaclonal variation (Linacero and Vazquez 1993, Ferreira et al. 2006a, Borchert et al. 2007, Jin et al. 2008).

Somaclonal variants are considered undesirable if clonal materials are required (Vajrabhaya 1977). Nevertheless, the ability to generate variations in a control manner could be beneficial for crop improvement (Gould 1986). However, somaclonal

variation occurs randomly (Larkin and Scowcroft 1981) and the mechanism of somaclonal variation is poorly understood (Puente et al. 2008). Possible mechanisms leading to somaclonal variation have been proposed. Somaclonal variation could be arise from genetic changes such as gene mutation (Dennis et al. 1987), a change in chromosome numbers (Lewis-Smith et al. 1990), the action of transposable elements and DNA methylation (Jaligot et al. 2000).

The frequency of somaclonal variation has been suggested to be affected by factors such as culture medium (Liscum and Hangarter 1991, Jin et al. 2008), the duration of the culture (Ezura and Oosawa 1994), genotype of the original explants (Brar and Jain 1998) and the source of explant (shoot tip, leaf, floral bud or root) (Vasil 1987, Linacero and Varquez 1993). The presence of plant growth regulator in the culture medium has an effect on the occurrence of somaclonal variants in cultures (Brar and Jain 1998). For instance, the addition of auxin, such as 2,4-D or IAA, to the culture medium resulted in an increase in the frequency of somaclonal variants in cotton and *Petunia* cultures (Liscum and Hangarter 1991, Jin et al. 2008). Somaclonal variation in the micro-propagated oil palm cultures was suspected to be due to the presence of cytokinins in the culture medium (Besse et al. 1992). These plant growth regulators may act as mutagens to the explants (Brar and Jain 1998).

The age of culture also influences somaclonal variation. In many long-term callus or cell cultures, a reduction or a complete loss of regeneration ability was generally observed (Brar and Jain 1998, Borchert et al. 2007). Muller et al. (1990) reported that the frequency of somaclonal variation increased with the duration of culture. Deletion of sections of the chloroplast genome of rice was observed in prolonged culture (Kawata et al. 1995). Furthermore, increased in ploidy of the somaclones were

reported in the *Quercus robur* after several years of continuous culture (Endemann et al. 2001).

Genotype also has an effect on somaclonal variation regardless of the regeneration mode (Bebeli et al. 1988). Among species or among genotypes within a species, differences in the frequency of variations have been observed (Brar and Jain 1998). Genotypes that carried transposable elements were more prone to somaclonal variation as compared to those without (Peschke and Phillips 1991).

Lastly, the source of explant has been found to influence the type and degree of somaclonal variation (Jain et al. 1998a, b). Plant regeneration is based on the concept of cell totipotency, so variations in the cells of explants hold the potential to result in the occurrence of somaclonal variants in cultures. For instance, explants that exhibit endopolyploidy would contain a mixture of cells of varying ploidy levels and shoots with varying ploidy levels might be regenerated from these explants (Lim and Loh 2003). Thus culturing of explants with cells of different ploidy levels might be a cause of somaclonal variation. An insight to the endopolyploidy profiles of plant tissues would be useful in providing a possible explanation to the role of endoreduplication in somaclonal variation.

2.7 *Dendrobium*

Orchids are monocots and belong to the family Orchidaceae of the order Orchidales. The orchid family is probably the largest in the plant kingdom (Arditti 1992) with about 750 different genera, 25,000 species and more than 30,000 hybrids (Hew and Yong 2004). Orchids can grow in two ways, namely sympodial and monopodial (Hew and Yong 2004). The growth of the shoot of sympodial orchid terminates in a flower

or inflorescence and continues its growth by laterally budding. However, in monopodial orchid, growth of shoot is continuous (Hew and Yong 2004). Orchid flowers are zygomorphic (symmetrical about a single plane). Each flower consists of a dorsal sepal, two lateral sepals, two petals, a labellum (modified petal), column (a coalescence of both male and female reproductive organs) and pedicel (Fig 2.3) that is connected to the pseudobulb or inflorescence stalk (Hew and Yong 2004).

Dendrobium is one of the largest genera in the family of Orchidaceae. It comprises of more than 1,000 species and distributed throughout Southeast Asia, Japan, Australia and the Pacific Islands (Kamemoto et al. 1999). The plants can be epiphytic or lithophytic with swollen stems or pseudobulbs. Its juvenile phase ranges from 2 to 5 years (Fadelah 2006, Sim et al. 2008). Cytological and ploidy studies on some *Dendrobium* species and hybrids showed that diploid *Dendrobium* generally had 38 chromosomes (Tanaka and Kamemoto 1984) and endoreduplication had been reported in some *Dendrobium* species (Jones and Kuehnle 1998).

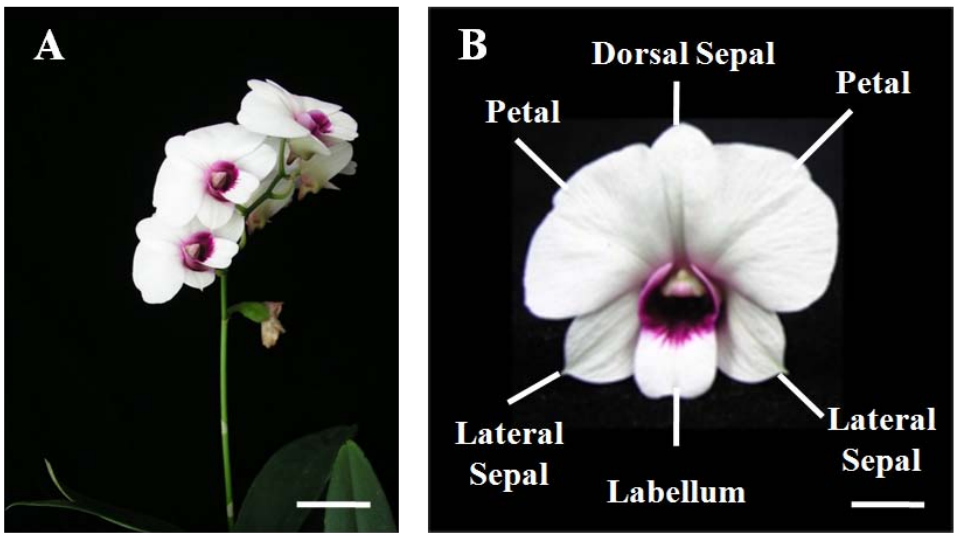

Fig 2.3. Flowers of greenhouse-grown *D.* Chao Praya Smile plants.

A: An inflorescence stalk, bar 2.33 cm.

B: Flower anatomy, bar 1.0 cm.

There is an increase in the popularity and demand of orchids (Winkelmann et al. 2006). Micro-propagation of orchids (Nayak et al. 2002, Park et al. 2002, Kuo et al. 2005, Martin and Madassery 2006) has been developed to meet the demand. Shoot tips (Roy et al. 2007), axillary buds (Ferreira et al. 2006b) and leaves (Martin and Madassery 2006) of *Dendrobium* are often used as explants for micro-propagation. High frequency *in vitro* flowering system has also been developed in *Dendrobium* hybrids, *D.* Madam Thong-In (Sim et al. 2007) and *D.* Chao Praya Smile (Hee et al. 2007). This allows early assessment (from 2-3 years to 3-5 months) of flower characteristics such as size, shape, tones and variation of colours (Hee et al. 2007, Sim et al. 2007). Furthermore, using the *in vitro* flowering cultures, studies on the effect of endogenous cytokinin on flowering and the identification of flower transition genes in *D.* Madam Thong-In were carried out (Yu et al. 2000, Yu and Goh 2000, Sim et al. 2008). Floral identity genes have also been isolated and studied in *D. crumenatum* (Xu et al. 2006). Other physiological and developmental studies of *Dendrobium* species and hybrids had also been reported (Goh et al. 1992, Stern et al. 1994, Carlsward et al. 1997, Vellupillai et al. 1997, Lopez and Runkle 2005, Ferreira et al. 2006b, Zha et al. 2007, He and Woon 2008, Yen et al. 2008).

In *D.* Second Love, it has been found that using protocorm-like bodies as explants for micro-propagation would lead to somaclonal variation which affected the clonal propagation of high quality hybrids (Ferreira et al. 2006a). It is speculated that endoreduplication might be one of the possible causes of somaclonal variation (Evans 1989, Lewis-Smith et al. 1990, Lim and Loh 2003). Therefore, the study of endoreduplication would provide a better understanding of the nature of the tissues that are often used in micro-propagation and aid our understanding of somaclonal variation in orchids.

In orchids, endoreduplication has been observed in leaves (Jones and Kuehnle 1998, Lim and Loh 2003, Yang and Loh 2004), root tips (Jones and Kuehnle 1998, Lim and Loh 2003, Yang and Loh 2004), perianth (Mishiba and Mii 2000, Lim and Loh 2003, Yang and Loh 2004) and protocorms (Alvarez 1968, Nagl 1972, Lim and Loh 2003). Systemic control of endoreduplication has also been reported in *V.* Miss Joaquim (Lim and Loh 2003) and *Spathoglottis plicata* (Yang and Loh 2004). However, detail study on the systemic control of endoreduplication in *Dendrobium* hybrid has yet to be investigated. With the high frequency *in vitro* flowering system that is developed in *D.* Chao Praya Smile, analyses of the endoreduplication profiles throughout the development of aseptically-grown *Dendrobium* seedlings are made possible.

2.8 *Anthurium andraeanum*

Anthurium is a monocot and belongs to the family Araceae. It is the largest and most morphologically diverse genus of Araceae, consisting of more than 1000 species (Martin et al. 2003). It is also one of the most important horticultural genera in the world (Matsumoto et al. 1998). In the global market, anthurium is second only to orchid among the tropical cut flower (Dufour and Guerin 2003). *A. andraeanum* is one of the highly cultivated anthurium (Dufour and Guerin 2003). Various herbaceous perennial ornamental species of *Anthurium* such as *A. andraeanum* and *A. scherzerianum* are commonly cultivated for their highly-prized flowers (Martin et al. 2003) and hybridisation of anthurium is usually limited to intra-specific hybridisations within *A. andraeanum* and *A. scherzerianum* (Sheffer and Kamemoto 1976a).

Each anthurium flower consists of a modified leaf (spathe) that subtends a cylindrical inflorescence called spadix (Fig 2.4) (Dufour and Guerin 2003). Unlike orchid,

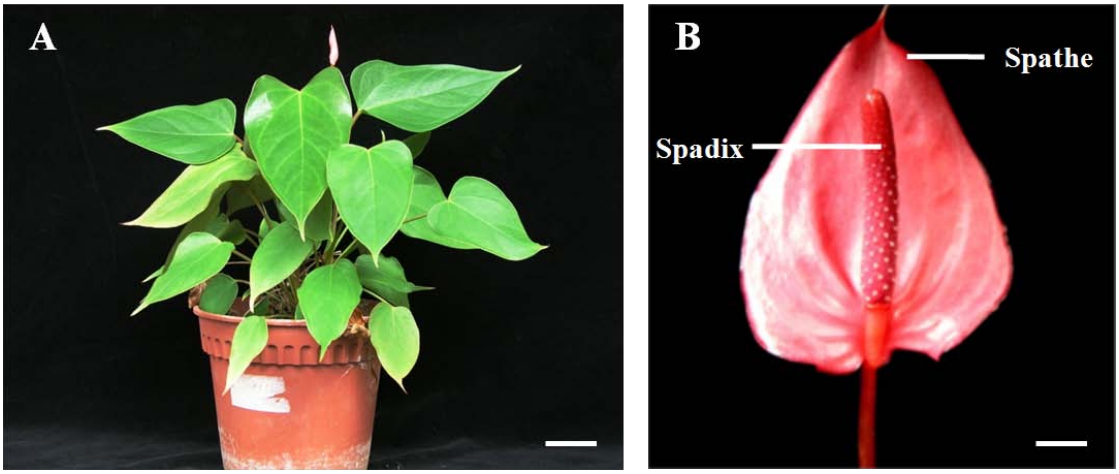

Fig 2.4. *A. andraeanum* cv 'Red Hot' plants.

A: Greenhouse-grown plants, bar 3.99 cm.

B: Flower anatomy, bar 1.0 cm.

anthurium exhibits both monopodial and sympodial growth within the same plant. During the juvenile and vegetative growth phase, it exhibits monopodial growth and the duration of monopodial phase is variety dependent (Dufour and Guerin 2003). During the adult stage, the plant has a sympodial phase with flower produced for each leaf (Dufour and Guerin 2003).

In *A. andraeanum*, several *in vivo* studies (Imamura and Higaki 1988, Dai and Paull 1990, Henny and Hamilton 1992, Hew et al. 1994, Dufour and Guerin 2003) have been carried out to investigate the respiration of flowers and factors affecting its growth and flowering. However, *in vitro* study of *A. andraeanum* is lacking and few cytological studies have been done on anthurium. Random amplified polymorphic DNA markers have been used to determine the genetic relationships of morphologically similar cultivars of potted *A. andraeanum* and other anthurium species (Ranamukhaarachchi et al. 2001). The chromosome numbers in several anthurium species studied varied, but majority of the species had chromosome number

of 30 (Sheffer and Kamemoto 1976b, Sheffer and Croat 1983). However, the number of chromosome in *A. andraeanum* is not investigated and cytological study of *A. andraeanum* is limited.

Anthurium can be propagated vegetatively or via seed germination. Propagation through seeds is difficult because some of the taxa fail to develop viable seeds due to incompatibility (Sheffer and Kamemoto 1976a). When pollination is successful, each berry (fruit) only contains 1 to 2 seeds which have low germination rate. Moreover, it may take 3 years from seed to bloom (Pierik et al. 1974). Hence, vegetative propagation is desirable. However, the conventional vegetative propagation of anthuriums by separating young plants from parents is time consuming and will take years to develop commercial quantities of the elite clone. Micro-propagation is an attractive alternative to propagate these plants in large quantity at a faster rate (Pierik et al. 1974). Micro-propagation of anthuriums was first reported by Pierik et al. (1974) and further refined by others (Kunisaki 1980, Kuehnle and Sugii 1991, Teng 1997, Martin et al. 2003). It has been shown that age and genotype of the explants would influence plant regeneration of *A. andraeanum* (Geier 1986). Micro-propagation allows a large number of anthurium plantlets to be produced from a small section of a piece of leaf or shoot tip. These micro-propagated plantlets are free from micro-organisms. Moreover, these plantlets could also be genetically modified to make them more resistance to some of the common plant diseases (Chen et al. 1997). The induction of somaclonal variation in the micro-propagated plants might also be a useful tool to improve the quality of horticulturally important plants (Brar and Jain 1998) such as *A. andraeanum*.

A possible cause of somaclonal variation is endoreduplication (Evans 1989, Lewis-Smith et al. 1990). However, nothing is known about endoreduplication in *A. andraeanum*. Thus, establishing the endoreduplication profiles of greenhouse-grown and micro-propagated *A. andraeanum* plants would be insightful and enhanced an understanding to the nature of tissues used for micro-propagation and the cytological and physiological aspects of anthurium. It might also provide an insight into the relation between somaclonal variation and endoreduplication.

Chapter 3 Systemic endopolyploidy in *D.* Chao Praya Smile

3.1 Introduction

Dendrobium is one of the largest genera in the family of Orchidaceae (Kamemoto et al. 1999). The genus comprises of more than 1,000 species and is distributed throughout Southeast Asia, Japan, Australia and the Pacific Islands (Kamemoto et al. 1999). Cytological studies have shown that diploid *Dendrobium* generally has 38 chromosomes per nucleus (Tanaka and Kamemoto 1984).

In *Dendrobium*, micro-propagation has been widely used to produce large number of true-to-type plants from the elite hybrids (Nayak et al. 2002, Martin and Madassery 2006, Arditti 2008). Unfortunately, not all the plants produced through micro-propagation were found to be true-to-type (Ferreira et al. 2006a). Variations have been reported among plants regenerated from tissue cultures of orchids (Vajrabhaya 1977, Ferreira et al. 2006a, Chen et al. 2008). A possible cause of the observed somaclonal variants could be the pre-existing genetic differences in somatic cells of the initial explants (Evans 1989). For instance, in tomato, the frequency of variants in the regenerants was positively correlated to the percentage of polyploid cells in the initial explants (Van den Bulk et al. 1990).

Different parts of the *Dendrobium* plants have been used as explants for micro-propagation and these include the shoot tips (Roy et al. 2007, Arditti 2008), axillary buds (Ferreira et al. 2006b, Arditti 2008), leaves (Martin and Madassery 2006, Arditti 2008), pseudobulb segments (Nayak et al. 1997, Arditti 2008) and floral stalks (Arditti 2008). However, very little is known about the ploidy variations in different tissues of *Dendrobium* plants during their development. The understanding of the degree of

endopolyploidy in the explant will therefore be beneficial for the maintenance of the original ploidy level in culture (Evans 1989, Kudo and Kimura 2001a).

Previously, endopolyploidy profiles of different organs and at different developmental stages of *Vanda* Miss Joaquim (Lim and Loh 2003), a monopodial orchid hybrid, and *Spathoglottis plicata* (Yang and Loh 2004), a terrestrial orchid, have been analysed. Jones and Kuehnle (1998) had made some examinations of the ploidy level in leaves, pollenia and to a lesser extent, roots in four *Dendrobium* species. As most commercially important dendrobiums are hybrids and not species, a better insight to their cellular ploidy profiles in different organs would be useful in understanding the nature of tissues used for micro-propagation. In addition, method to induce early *in vitro* flowering in an orchid hybrid, *D.* Chao Praya Smile, has been developed (Hee et al. 2007). It will, therefore, be interesting to investigate the endopolyploidy profiles of these cultures at different developmental stages.

Hence, the objectives of this chapter are: (1) to analyse the occurrence of multiploid cells in different tissues in greenhouse-grown plants and aseptically-grown seedlings of a commercial hybrid, *D.* Chao Praya Smile, and (2) to determine the pattern of endopolyploidy throughout the development of the plant. The patterns of endopolyploidy in different organs of greenhouse-grown plants and aseptically-grown seedlings of *D.* Chao Praya Smile were followed from seed germination to different developmental stages.

3.2 Materials and methods

3.2.1 Plant materials, culture media and culture conditions

D. Chao Praya Smile (*D.* Pinky × *D.* Kiyomi Beauty) plants were grown in pots in the greenhouse in the Department of Biological Sciences, National University of Singapore, at 28 ± 4 ºC with natural lighting of intensity 90.4 ± 12.4 µE m^{-2} s^{-1}.

Seeds and protocorms

Three-month-old seed pods were washed with detergent, surface sterilised in 20 % (v/v) Clorox^{TM} solution with a drop of Tween 20 for 15 min with constant agitation. The seed pods were then rinsed twice with autoclaved water and cut into halves. Seeds were germinated aseptically in 50 ml Knudson C medium (KC, Knudson 1946) supplemented with 2 % (w/v) sucrose and 15 % (v/v) coconut water at pH 5.3 in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm for proliferation. The cultures were maintained at 25 ºC under 16 hours illumination of intensity 45.9 ± 1.3 µE m^{-2} s^{-1}. Flow cytometric analyses of seeds and protocorms were conducted every 3 weeks for 12 weeks, starting from week 0 of germination.

Greenhouse-grown plants

Shoot tips (0.1- to 0.2-cm in length) and axillary buds (0.1- to 0.2-cm in diameter) (see Fig 3.1A) from greenhouse-grown plants with six fully expanded leaves were used. Each replicate was made up of 4 samples and triplicates were conducted.

Greenhouse-grown plants of about 16-cm tall (measured from the youngest leaf tip to the bottom of pseudobulb) with unopened and fully expanded leaves were used for flow cytometric analyses. The leaves were numbered from the shoot apex downwards,

with the youngest leaf as first leaf (normally concealed by the second leaf) (see Fig 3.2). For the 1st and 6th leaves, the entire leaf was analysed as one sample due to their smaller size. For the rest (2nd to 5th leaves), the tip (distal lamina), mid (middle lamina) and base (proximal lamina) sections of the leaves were analysed separately. For roots shorter than 1.5-cm, they were analysed as one sample. For roots of 6-cm in length, the tip and base sections were analysed separately. As for the flower, the column (with pollenia removed), pedicel, labellum, petals, dorsal and lateral sepals were analysed separately. Floral stages were investigated 1 day before anthesis and 5, 15 and 25 days after anthesis (Fig 3.3). Anthesis was taken as the time at which the flower bud fully opened.

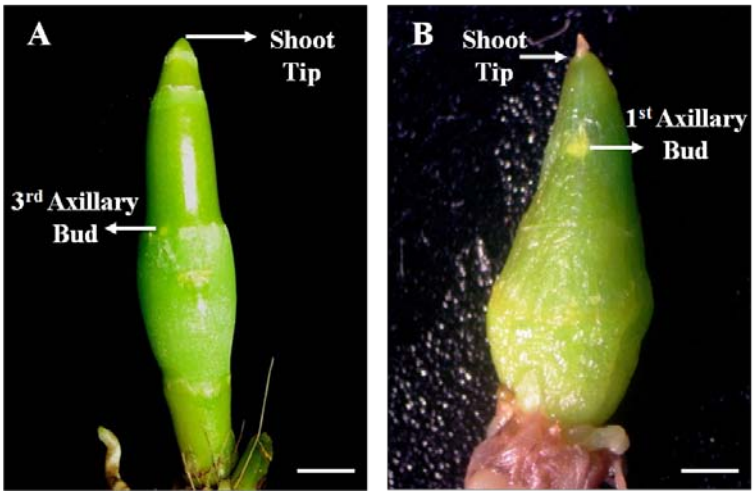

Fig 3.1. Pseudobulbs of *D.* Chao Praya Smile.

A: From greenhouse-grown plant, bar: 0.5 cm.

B: From aseptically-grown seedling, bar: 0.2 cm.

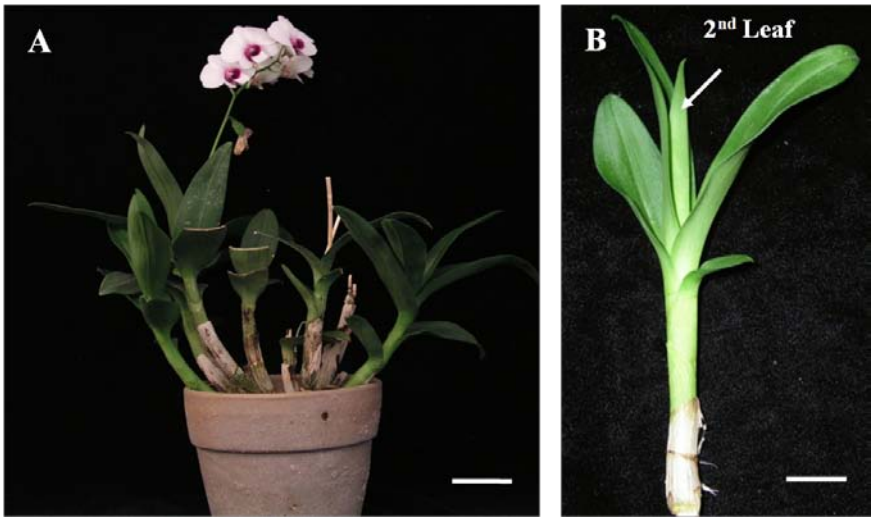

Fig 3.2. Greenhouse-grown *D.* Chao Praya Smile plants.

A: Potted plants, bar: 2.4 cm.

B: A shoot taken from a greenhouse-grown plant showing position of the second leaf (first leaf was concealed), bar: 2.7 cm.

Fig 3.3. Flowers of greenhouse-grown *D.* Chao Praya Smile plants at different stages of anthesis (Days before (-) and after (+) anthesis), bar: 1.0 cm.

Aseptically-grown seedlings

The aseptically-grown seedlings were maintained at 25 ºC under 16 hours illumination of intensity 45.9 ± 1.3 µE m^{-2} s^{-1}. Shoot tips (0.1- to 0.2-cm in length) and axillary buds (0.1- to 0.2-cm in diameter) (Fig 3.1B) from seedlings (about 10-month-old) with five fully expanded leaves were used for flow cytometric analyses. Each replicate was made up of 4 samples and triplicates were conducted.

For the analyses of leaves, leaves from the 10-month-old seedlings were numbered from the shoot apex downwards, with the youngest fully expanded leaf as 1st leaf (Fig 3.4A). For the 5th leaf, the entire leaf was analysed as a whole due to its small size. The tip, mid and base sections of the other leaves (1st to 4th leaves) were analysed separately. Analysis of unopened leaf was done on the 1st unopened leaf of 7-month-old seedlings (Fig 3.4B). Roots of 1.5-cm were analysed as one sample. The tip and base sections of 6-cm long root were analysed separately.

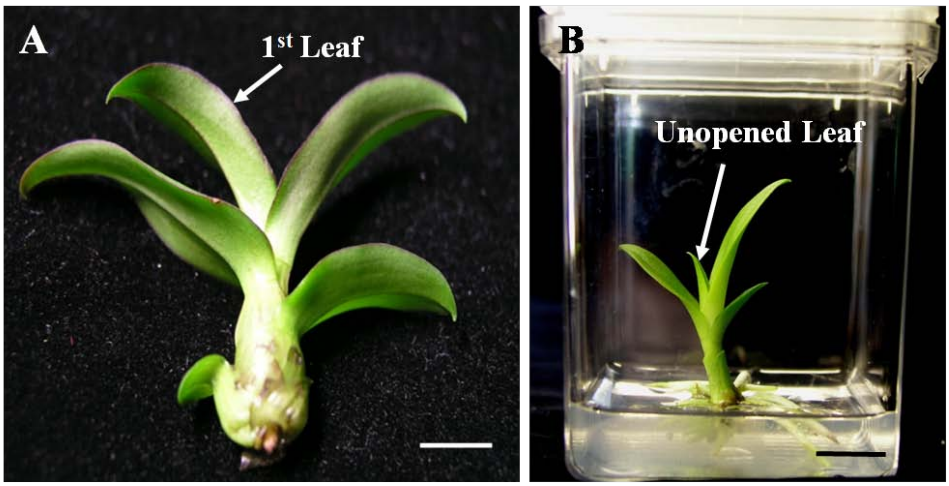

Fig 3.4. Aseptically-grown *D.* Chao Praya Smile seedlings.

A: Ten-month-old aseptically-grown seedling with five fully expanded leaves, bar: 1.2 cm.

B: Seven-month-old aseptically-grown seedling with unopened leaf, bar: 1.5 cm.

Induction of *in vitro* flowering

Eight-week-old protocorms were transferred to 50 ml of liquid modified KC medium, according to Hee et al. (2007), in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm for proliferation. The liquid medium was also supplemented with 2 % (w/v) sucrose, 15 % (v/v) coconut water and 11.1 µM BA (Hee et al. 2007). After three rounds of subculturing in the liquid medium at 3-week intervals, the seedlings were transferred to two layer modified KC medium in Magenta GA7^{TM} containers (Hee et al. 2007, Sim et al. 2007). The two-layer culture media consisted of 50 ml of gelrite-solidified medium topped with a layer of liquid medium of the same volume and composition (Hee et al. 2007). All cultures were incubated at 25 ºC under 16 hours illumination of intensity 45.9 ± 1.3 µE m^{-2} s^{-1}. Flowers induced from the seedlings were used for flow cytometric analyses. Different flower parts were analysed separately.

3.2.2 Nuclei isolation for flow cytometric analyses

All samples, excluding seeds, were washed with de-ionised water and dried before nuclei isolation. All steps of nuclei isolation were carried out on ice. The sample was fixed in 3-5 ml of fixation buffer containing (mM): Tris (10), Na_2EDTA (10), NaCl (100) and 0.1 % (v/v) Triton X-100, pH 7.5 (20 min for perianth and 30 min for other sample types). The sample was then washed once with 2 ml extraction buffer containing (mM): $MgSO_4.7H_2O$ (10), KCl (50), HEPES (5), 1 mg ml^{-1} dithiothreitol and 0.25 % (v/v) Triton X-100 (Arumuganathan and Earle 1991). Tissue samples were chopped with a sterile doubled-sided razor blade in extraction buffer at 0.08 g tissue to 1 ml extraction buffer ratio. For seeds and 3-week-old protocorms, they were gently pressed in the buffer with a clean glass rod to release the nuclei. After incubation for 1

hour in the extraction buffer, the nuclei suspension was filtered through a 40-µm nylon cell strainer and kept on ice until flow cytometric analysis was carried out.

3.2.3 Flow cytometric analyses

Nuclei staining was carried out by incubating 500 µl of nuclei suspension with 25 µl of 5 mg ml^{-1} propidium iodide and 50 µl of 10 µg ml^{-1} DNase-free RNase for 30 min at room temperature. Each sample was filtered through 40-µm nylon filter prior to analysis. Flow cytometric analyses were performed using a Coulter EPICS® Altra ESP flow cytometer (Coulter, Miami, Florida, USA) with laser at 488 nm excitation. For each sample, 10 000 nuclei were analysed, where C is the nuclear DNA content of haploid genome. The 2C peak of fluorescence produced by nuclei extracted from the tip of the 4^{th} leaf of mature greenhouse-grown plant was used to estimate the standard peak position of 2C nuclei. Data were analysed with software WINMDI (version 2.8). Quadruplicates were done for all experiments unless otherwise stated.

3.2.4 Statistical analyses

All percentage data were analysed with one-way analysis of variance (ANOVA) and Tukey's test at 95 % confidence level was performed for multiple pair-wise comparisons. Data were arcsine-transformed to meet assumptions of ANOVA (normality and homogeneity of variance) and analysed with Minitab™ 15.

3.3 Results

3.3.1 Endopolyploidy in seeds and protocorms cultured in liquid basal KC medium

Nuclei extracted from the seeds freshly isolated from seed pods (week 0) were found to have only 2C DNA content (Table 3.1; Fig 3.5A). After 3 weeks of culture, the seeds developed into small protocorms (Fig 3.5B). During protocorm development, endopolyploidy occurred progressively. Nuclei of five ploidy levels corresponding to 2C, 4C, 8C, 16C and 32C were found in the 3-week-old protocorms (Table 3.1). In 3-week-old protocorms, the mean frequency of 2C nuclei (54.06 %) was significantly higher, while the mean frequency of 8C nuclei (10.56 %) was significantly lower than that of the older protocorms (Table 3.1).

After 9 weeks of culture, the protocorms consisted of nuclei with up to 64C DNA content (Table 3.1). At 9^{th} and 12^{th} weeks of culture, some protocorms developed leaves (Figs 3.5D and F). Majority of the protocorms, however, remained leafless (Figs 3.5E and G). In both 9- and 12-week-old cultures, the protocorms with leaves were found to contain more 2C nuclei than the leafless protocorms. The mean frequencies of the various nuclei (up to 32C) in the protocorms of the same type from 9- and 12-week-old cultures showed no significant differences (Table 3.1).

Table 3.1. Mean proportion of nuclei of various DNA contents (C-values) from *D.* Chao Praya Smile seeds and protocorms cultured in liquid basal KC medium during development. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Weeks of Culture	Type of Culture	Mean Proportion of Nuclei (%) ± SE					
		2C	4C	8C	16C	32C	64C
0	Seeds	100 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3	Protocorms	54.06 ± 0.34^{c}	30.06 ± 0.39^{b}	10.56 ± 0.31^{c}	4.02 ± 0.24^{b}	1.30 ± 0.15^{b}	0.00 ± 0.00^{a}
6	Protocorms	46.97 ± 0.46^{b}	31.84 ± 0.36^{bc}	14.08 ± 0.25^{b}	5.35 ± 0.35^{bd}	1.76 ± 0.10^{b}	0.00 ± 0.00^{a}
9	Protocorms	34.03 ± 0.99^{d}	31.50 ± 1.48^{bc}	20.44 ± 0.91^{d}	11.19 ± 0.96^{e}	2.84 ± 0.74^{b}	0.00 ± 0.00^{a}
	Protocorms with Leaves	40.83 ± 1.58^{b}	30.71 ± 0.80^{b}	17.35 ± 0.76^{bd}	8.27 ± 0.76^{cde}	2.37 ± 0.66^{b}	0.47 ± 0.18^{b}
12	Protocorms	32.02 ± 1.03^{d}	32.28 ± 0.72^{bc}	20.48 ± 0.55^{d}	12.00 ± 0.73^{e}	3.22 ± 0.56^{b}	0.00 ± 0.00^{a}
	Protocorms with Leaves	40.85 ± 2.72^{b}	34.09 ± 0.26^{c}	16.35 ± 1.57^{b}	6.92 ± 1.60^{bc}	1.79 ± 0.33^{b}	0.00 ± 0.00^{a}

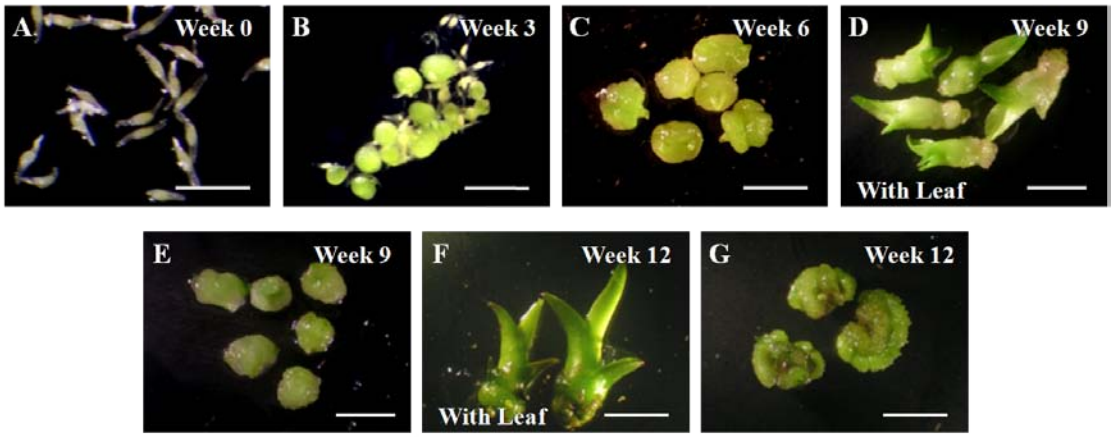

Fig 3.5. Development of seeds and protocorms of *D.* Chao Praya Smile in liquid basal KC medium over 12 weeks.

A: Seeds at week 0, bar: 0.05 cm.

B: 3-week-old protocorms, bar: 0.3 cm.

C: 6-week-old protocorms, bar: 0.3 cm.

D , E: 9-week-old protocorms, bar: 0.5 cm.

F , G: 12-week-old protocorms, bar: 0.8 cm.

Note that in 9- and 12-week-old cultures, some protocorms developed leaves (**D**, **F**).

3.3.2 Endopolyploidy in the shoot tips, axillary buds and pseudobulbs

Greenhouse-grown plants

The shoot tips of greenhouse-grown plants were made up of nuclei from 2C to 8C DNA content (Table 3.2; Fig 3.1A). No significant difference was found in the mean proportions of 2C and 4C nuclei in the shoot tips compared to those in the 1st axillary buds (Table 3.2). The mean frequency of 8C nuclei in the 1st axillary buds was 7.44 %, which was significantly higher than that in the shoot tips (4.83 %) (Table 3.2). Some 16C nuclei (with mean frequency of about 1 %) were detected in the axillary buds and pseudobulbs, but not in the shoot tips (Table 3.2).

Aseptically-grown seedlings

Shoot tips and 1st axillary buds of aseptically-grown seedlings consisted of nuclei of up to 8C DNA content (Table 3.3; Fig 3.1B). 16C nuclei were detected in the lateral shoots and pseudobulbs. The distributions of the various nuclei in the shoot tips were not significantly different from those of the 1st axillary buds (Table 3.3). In the 0.5-cm lateral shoots developed from axillary buds, the mean proportion of 16C nuclei (0.84 %) was higher than those of the shoot tips and 1st axillary buds (Table 3.3). In the pseudobulbs, the mean frequency of 2C nuclei was significantly lower, while the mean frequencies of 8C and 16C nuclei were found to be significantly higher as compared to those from the shoot tips, axillary buds and lateral shoots (Table 3.3).

Table 3.2. Mean proportion of nuclei of various DNA contents (C-values) from the shoot tip, axillary bud and pseudobulb tissues of greenhouse-grown *D.* Chao Praya Smile plants (For shoot tips and axillary buds, triplicates were conducted and results are presented as means of triplicates). Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Plant Part	Mean Proportion of Nuclei (%) ± SE			
	2C	4C	8C	16C
Shoot Tip	53.88 ± 0.83^{a}	41.29 ± 1.18^{ab}	4.83 ± 0.35^{a}	0.00 ± 0.00^{a}
1st Axillary Bud	48.79 ± 1.24^{a}	43.21 ± 1.45^{ab}	7.44 ± 0.12^{b}	0.56 ± 0.31^{a}
3rd Axillary Bud	45.31 ± 2.60^{a}	44.94 ± 3.07^{a}	8.89 ± 1.05^{b}	0.86 ± 0.20^{a}
Pseudobulb	53.66 ± 3.12^{a}	35.91 ± 1.55^{b}	9.48 ± 1.39^{b}	0.95 ± 0.30^{a}

Table 3.3. Mean proportion of nuclei of various DNA contents (C-values) from the shoot tip, axillary bud and pseudobulb tissues and 0.5-cm lateral shoots of aseptically-grown *D.* Chao Praya Smile seedlings. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Plant Part	Mean Proportion of Nuclei (%) ± SE			
	2C	4C	8C	16C
Shoot Tip	70.45 ± 3.92^{a}	28.12 ± 3.88^{a}	1.43 ± 0.19^{a}	0.00 ± 0.00^{a}
1st Axillary Bud	61.00 ± 6.37^{a}	36.13 ± 5.81^{ab}	2.87 ± 0.62^{ab}	0.00 ± 0.00^{a}
Lateral Shoot	64.63 ± 2.21^{a}	29.83 ± 1.74^{a}	4.70 ± 0.69^{b}	0.84 ± 0.07^{b}
Pseudobulb	33.67 ± 1.13^{b}	50.79 ± 1.68^{b}	14.25 ± 0.87^{c}	1.29 ± 0.18^{c}

3.3.3 Endopolyploidy in the leaves

Greenhouse-grown plants

Endopolyploidy profiles of developing and matured leaves were shown in Table 3.4. Leaves from greenhouse-grown plants contained nuclei of up to 16C DNA content (Table 3.4). The unopened 1st leaf (youngest leaf) of a vegetatively growing shoot contained 56.02 % of 2C nuclei, while the mean proportion of 8C nuclei was about 4 % (Table 3.4). As the leaves developed, nuclei of 16C DNA content were detected from the 3rd leaf onwards (Table 3.4). The 6th leaf (oldest leaf) contained only a mean proportion of 15.32 % 2C nuclei and its 8C and 16C nuclei accounted for up to 28.06 and 2.59 % of the total nuclei population analysed, respectively (Table 3.4). The 6th leaf was found to contain significantly less 2C nuclei and more 8C nuclei than the 1st leaf (Table 3.4).

For the 2nd to 5th leaves, endopolyploidy in the tip, mid and base sections were analysed (Table 3.4). The mean proportion of 2C nuclei in the tip of the 2nd leaf was 24.66 % and was significantly lower than that in its base (59.88 %) (Table 3.4). Furthermore, the mean frequencies of 4C and 8C nuclei in the tip of 2nd leaf (55.13 and 20.21 %, respectively) were found to be significantly higher than those in its base (34.06 and 6.06 %, respectively) (Table 3.4).

Similarly, the tips of the 3rd and 4th leaves were found to contain significantly less 2C nuclei and more 8C nuclei than their bases (Table 3.4). In the 4th leaf, nuclei with 16C DNA content were detected in the tip and mid sections, but not the base section (Table 3.4). For the 5th leaf, no significant difference in the proportions of nuclei with 2C, 4C, 8C and 16C DNA content was observed among the tip, mid and base sections. These

Table 3.4. Mean proportion of nuclei of various DNA contents (C-values) from different parts of greenhouse-grown *D.* Chao Praya Smile leaves during vegetative development. The 1st leaf (starting from the shoot tip was unopened and the youngest). Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Position of Leaf	Leaf Part	Mean Proportion of Nuclei (%) ± SE			
		2C	4C	8C	16C
1st	Whole	56.02 ± 2.41^{a}	39.74 ± 1.70^{ac}	4.24 ± 1.02^{a}	0.00 ± 0.00^{a}
2nd	Tip	24.66 ± 4.17^{bc}	55.13 ± 0.80^{ab}	20.21 ± 4.76^{bd}	0.00 ± 0.00^{a}
	Mid	26.45 ± 3.53^{bc}	56.47 ± 1.83^{b}	17.08 ± 3.03^{bce}	0.00 ± 0.00^{a}
	Base	59.88 ± 4.44^{a}	34.06 ± 3.71^{c}	6.06 ± 2.32^{ac}	0.00 ± 0.00^{a}
3rd	Tip	20.26 ± 2.52^{bc}	51.06 ± 1.92^{abd}	28.68 ± 1.91^{ed}	0.00 ± 0.00^{a}
	Mid	26.20 ± 2.30^{bc}	50.45 ± 2.37^{abd}	22.47 ± 2.73^{bde}	0.88 ± 0.88^{ab}
	Base	54.41 ± 5.74^{a}	39.67 ± 4.25^{ac}	5.92 ± 1.65^{ac}	0.00 ± 0.00^{a}
4th	Tip	13.56 ± 2.56^{c}	51.30 ± 4.42^{abd}	32.32 ± 4.46^{de}	2.82 ± 0.73^{b}
	Mid	23.51 ± 3.04^{bc}	50.99 ± 4.48^{abd}	25.12 ± 3.51^{de}	0.38 ± 0.24^{ab}
	Base	39.54 ± 7.11^{ab}	50.79 ± 4.96^{abd}	9.67 ± 2.23^{ab}	0.00 ± 0.00^{a}
5th	Tip	17.25 ± 2.58^{c}	45.17 ± 1.70^{abc}	35.45 ± 2.78^{d}	2.13 ± 0.57^{ab}
	Mid	15.60 ± 2.38^{c}	48.49 ± 2.14^{abc}	34.47 ± 3.86^{de}	1.44 ± 0.78^{ab}
	Base	19.63 ± 2.08^{c}	57.20 ± 0.85^{b}	22.71 ± 2.60^{bde}	0.46 ± 0.28^{ab}
6th	Whole	15.32 ± 4.11^{c}	54.03 ± 5.36^{ab}	28.06 ± 5.25^{de}	2.59 ± 2.06^{ab}

mean nuclei frequencies were not significantly different from those of the 6th leaf (Table 3.4).

Comparisons between the first leaves from plants at the early (before bolting) and late stages of the reproductive (4 months after bolting) phase were made (Table 3.5). No significant difference in the distributions of multiploid cells were observed among the tip, mid and base sections of the 1st leaves at these two stages (Table 3.5). However, the mean frequencies of 4C nuclei in the tip and base sections of leaf at the late reproductive phase (37.32 and 37.86 %, respectively) were found to be significantly lower than those in the tip and base sections of the leaf just before bolting (Table 3.5). Furthermore, the distributions of 16C nuclei in the tip and base sections of the leaf at the late reproductive phase (12.71 and 7.15 %, respectively) were found to be significantly higher than those in the tip and base sections of the leaf at the beginning of bolting (Table 3.5). Only leaf at the late reproductive phase contained nuclei with 32C DNA content (Table 3.5).

Aseptically-grown seedlings

The mean frequency of 2C nuclei in the unopened 1st leaf was 61.84 % and 2.3 % of the total nuclei population analysed was found to be 8C nuclei (Table 3.6). The unopened 1st leaf was found to contain significantly more 2C nuclei (about 27 % more) and less 8C nuclei (about 15 % less) than the 5th leaf (Table 3.6).

The 1st to 3rd leaves were found to contain nuclei of up to 8C DNA content. 16C nuclei were detected only in the 4th and 5th leaves (Table 3.6). The tip sections of the 3rd and 4th leaves contained less 2C nuclei than their base sections (Table 3.6).

Table 3.5. Mean proportion of nuclei of various DNA contents (C-values) from different parts of the first leaves of greenhouse-grown *D.* Chao Praya Smile taken from shoots just before bolting and 4 months after bolting. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Stage of Shoot	Parts of First Leaf	Mean Proportion of Nuclei (%) ± SE				
		2C	4C	8C	16C	32C
Before Bolting	Tip	20.80 ± 1.26^{ab}	51.83 ± 0.89^{a}	25.91 ± 1.72^{a}	1.46 ± 0.23^{ac}	0.00 ± 0.00^{a}
	Mid	22.12 ± 2.04^{ab}	47.17 ± 0.71^{ac}	28.96 ± 2.20^{ab}	1.75 ± 0.43^{ac}	0.00 ± 0.00^{a}
	Base	25.74 ± 1.95^{a}	49.01 ± 0.45^{a}	24.67 ± 1.98^{a}	0.59 ± 0.34^{a}	0.00 ± 0.00^{a}
Four Months After Bolting	Tip	18.01 ± 1.35^{ab}	37.32 ± 3.06^{b}	31.13 ± 0.87^{ab}	12.71 ± 3.63^{b}	0.83 ± 0.47^{a}
	Mid	16.59 ± 1.89^{b}	37.84 ± 3.72^{bc}	34.07 ± 2.03^{b}	10.94 ± 4.62^{bc}	0.56 ± 0.56^{a}
	Base	20.52 ± 2.17^{ab}	37.86 ± 1.18^{bc}	34.20 ± 1.32^{b}	7.15 ± 1.33^{bc}	0.27 ± 0.16^{a}

Table 3.6. Mean proportion of nuclei of various DNA contents (C-values) from different parts of aseptically-grown *D.* Chao Praya Smile leaves during vegetative development. The 1st (Opened) leaf represented the youngest fully expanded leaf from 10-month-old seedlings, while 1st (Unopened) leaf was obtained from 7-month-old seedlings with unopened leaves (Fig 3.4). Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Position of Leaf	Leaf Part	Mean Proportion of Nuclei (%) ± SE			
		2C	4C	8C	16C
1st (Unopened)	Whole	61.84 ± 2.87^{a}	35.85 ± 2.38^{a}	2.30 ± 0.57^{a}	0.00 ± 0.00^{a}
1st (Opened)	Tip	29.57 ± 0.36^{cdf}	57.73 ± 2.13^{c}	12.70 ± 2.38^{ba}	0.00 ± 0.00^{a}
	Mid	25.19 ± 2.41^{bd}	51.37 ± 3.09^{ac}	23.44 ± 4.39^{bc}	0.00 ± 0.00^{a}
	Base	29.97 ± 0.56^{bde}	54.25 ± 2.48^{ac}	17.78 ± 2.69^{b}	0.00 ± 0.00^{a}
2nd	Tip	25.76 ± 0.84^{bd}	54.01 ± 0.84^{ac}	20.23 ± 2.41^{bc}	0.00 ± 0.00^{a}
	Mid	22.17 ± 1.49^{bf}	50.46 ± 4.08^{ac}	27.37 ± 5.26^{bc}	0.00 ± 0.00^{a}
	Base	33.16 ± 1.77^{cd}	51.38 ± 0.98^{ac}	15.46 ± 2.36^{b}	0.00 ± 0.00^{a}
3rd	Tip	25.83 ± 2.33^{bd}	55.43 ± 2.61^{ac}	18.74 ± 3.98^{bc}	0.00 ± 0.00^{a}
	Mid	27.87 ± 2.08^{bc}	56.10 ± 2.93^{ac}	16.03 ± 4.21^{bc}	0.00 ± 0.00^{a}
	Base	34.59 ± 2.99^{ce}	50.12 ± 0.92^{ac}	15.29 ± 2.68^{b}	0.00 ± 0.00^{a}
4th	Tip	21.96 ± 1.41^{bf}	52.07 ± 3.31^{ac}	25.61 ± 3.92^{bc}	0.36 ± 0.36^{ab}
	Mid	18.88 ± 1.22^{b}	46.19 ± 4.54^{bc}	34.36 ± 5.00^{c}	0.57 ± 0.33^{abc}
	Base	36.66 ± 1.42^{c}	45.56 ± 1.86^{bc}	16.80 ± 1.79^{bc}	0.98 ± 0.58^{abc}
5th	Whole	34.86 ± 0.80^{ce}	45.53 ± 2.46^{bc}	17.22 ± 1.14^{bc}	2.39 ± 0.94^{c}

3.3.4 Endopolyploidy in the roots

Greenhouse-grown plants

More than half of the nuclei in the 1.5-cm root were 2C. The highest DNA content detected in the 1.5-cm root and the tip of the 6-cm root was 8C in a mean frequency of 4.17 and 9.27 %, respectively (Table 3.7). The mean frequency of 2C nuclei in the 1.5-cm root was 52.91 % and was significantly higher than that in the base section of the 6-cm root (37.92 %) (Table 3.7). 16C nuclei were detected in the base section of the 6-cm root (Table 3.7).

Aseptically-grown seedlings

Nuclei of up to 32C DNA content were detected in the roots of aseptically-grown seedlings; ranging from 0.5 to 1.4 % of the total nuclei population analysed (Table 3.8). The mean frequency of 2C nuclei in the tip of the 6-cm root (30.99 %) was significantly higher than that in its base (16.46 %). However, the tip of the 6-cm root was found to contain significantly less 8C nuclei (about 15 % less) than its base (Table 3.8).

Table 3.7. Mean proportion of nuclei of various DNA contents (C-values) from different parts of greenhouse-grown *D.* Chao Praya Smile roots during development (Triplicates were conducted and results are presented as means of triplicates). Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Length (cm)	Part	Mean Proportion of Nuclei (%) ± SE			
		2C	4C	8C	16C
1.5	Whole	52.91 ± 3.88[a]	42.92 ± 4.07[a]	4.17 ± 1.68[a]	0.00 ± 0.00[a]
6	Tip	49.04 ± 1.12[ab]	41.69 ± 0.94	9.27 ± 1.15[ab]	0.00 ± 0.00[a]
	Base	37.92 ± 5.06[b]	41.96 ± 1.74[a]	18.69 ± 3.72[b]	1.43 ± 0.26[b]

Table 3.8. Mean proportion of nuclei of various DNA contents (C-values) from different parts of aseptically-grown *D.* Chao Praya Smile roots during development. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Length (cm)	Part	Mean Proportion of Nuclei (%) ± SE				
		2C	4C	8C	16C	32C
1.5	Whole	18.09 ± 0.93[a]	39.97 ± 1.36[a]	33.22 ± 0.84[ab]	8.15 ± 1.49[a]	0.57 ± 0.19[a]
6	Tip	30.99 ± 2.58[b]	40.01 ± 1.15[a]	23.51 ± 2.17[b]	4.96 ± 0.71[a]	0.53 ± 0.04[a]
	Base	16.46 ± 1.39[a]	36.43 ± 0.80[a]	38.01 ± 2.52[a]	7.71 ± 1.40[a]	1.39 ± 0.41[a]

3.3.5 Endopolyploidy in the flowers

Greenhouse-grown plants

Flowers were found to exhibit endopolyploidy and the distributions of the multiploid cells were found not to be developmentally regulated (Table 3.9). From 1 day before anthesis to 25 days after anthesis, no significant change was observed in the endopolyploidy patterns in the floral tissues (Table 3.9). Nuclei with 2C and 4C DNA content accounted for over 80 % of the cells in all the floral tissues analysed (Table 3.9). In the column, the mean proportions of 8C nuclei from 1 day before anthesis to 25 days after anthesis ranged from 12.18 to 14.11 % and the mean proportion of the 16C nuclei was significantly higher than other floral parts (Table 3.9).

Aseptically-grown seedlings

Endopolyploidy was observed in complete flowers of aseptically-grown seedlings (Table 3.10; Fig 3.6A). There were no significant changes in the endopolyploidy patterns in the floral tissues at 5 and 15 days after anthesis (Table 3.10). The mean frequencies of 8C nuclei in the column at 5 and 15 days after anthesis were 12.05 and 14.27 %, respectively and were found to be higher than other floral tissues (Table 3.10).

Some flowers produced in the cultures were deformed. In such flowers, the dorsal sepal was absent and the flowers failed to open fully (Fig 3.6B). At 15 days after anthesis, nuclei with up to 8C DNA content were detected in all the tissues of the deformed flowers analysed (Table 3.11). 32C nuclei were only detected in the column of the deformed flowers at a mean frequency of 0.65 % (Table 3.11).

Table 3.9. Mean proportion of nuclei of various DNA contents (C-values) from different floral tissues of greenhouse-grown *D.* Chao Praya Smile plants during development. Different letters following the standard error within the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

Floral Part	Floral Stage	Mean Proportion of Nuclei (%) ± SE			
	Days Before (-) And After (+) Anthesis	2C	4C	8C	16C
Dorsal Sepal	- 1	41.44 ± 1.0^{abc}	53.35 ± 0.78^{acd}	4.70 ± 0.26^{ac}	0.51 ± 0.07^{a}
	+ 5	39.57 ± 0.36^{ad}	56.17 ± 0.85^{ad}	4.26 ± 0.72^{ac}	0.00 ± 0.00^{a}
	+ 15	41.68 ± 1.21^{abc}	53.77 ± 1.88^{acd}	4.55 ± 0.68^{ac}	0.00 ± 0.00^{a}
	+ 25	40.71 ± 2.68^{ac}	53.35 ± 2.90^{acd}	5.39 ± 0.71^{ac}	0.55 ± 0.07^{a}
Lateral Sepals	- 1	37.23 ± 0.50^{a}	56.70 ± 0.44^{a}	5.38 ± 0.30^{ac}	0.69 ± 0.16^{a}
	+ 5	41.01 ± 2.14^{ac}	54.56 ± 2.11^{ad}	4.43 ± 0.27^{ac}	0.00 ± 0.00^{a}
	+ 15	41.73 ± 1.80^{abc}	52.25 ± 1.37^{acde}	5.59 ± 0.58^{ac}	0.43 ± 0.08^{a}
	+ 25	42.37 ± 1.88^{abc}	52.14 ± 1.71^{acde}	4.97 ± 0.85^{ac}	0.52 ± 0.08^{a}
Petals	- 1	51.19 ± 0.65^{bfh}	43.60 ± 1.76^{beh}	4.21 ± 1.28^{ac}	1.00 ± 0.58^{a}
	+ 5	56.07 ± 1.84^{fh}	41.09 ± 1.40^{beg}	2.84 ± 0.46^{a}	0.00 ± 0.00^{a}
	+ 15	56.72 ± 1.14^{fh}	39.10 ± 2.24^{bfg}	4.18 ± 1.38^{ac}	0.00 ± 0.00^{a}
	+ 25	55.74 ± 0.98^{fh}	39.83 ± 1.48^{bfg}	3.97 ± 0.33^{ac}	0.46 ± 0.21^{a}
Labellum	- 1	48.69 ± 0.65^{cdf}	47.78 ± 0.79^{bd}	3.53 ± 0.28^{ad}	0.00 ± 0.00^{a}
	+ 5	51.21 ± 0.70^{cfh}	45.19 ± 0.54^{bc}	3.60 ± 0.43^{ad}	0.00 ± 0.00^{a}
	+ 15	55.48 ± 0.65^{fh}	41.20 ± 0.69^{bfg}	3.32 ± 0.10^{ad}	0.00 ± 0.00^{a}
	+ 25	50.27 ± 1.23^{cef}	45.59 ± 0.86^{bc}	4.14 ± 0.42^{ac}	0.00 ± 0.00^{a}
Column	- 1	40.95 ± 2.50^{ac}	42.75 ± 2.44^{bg}	14.11 ± 0.90^{be}	2.19 ± 0.21^{bc}
	+ 5	39.67 ± 1.12^{ad}	45.93 ± 1.32^{bc}	12.43 ± 1.97^{be}	1.97 ± 0.51^{bc}
	+ 15	41.24 ± 1.24^{ac}	44.01 ± 1.63^{beh}	12.18 ± 1.36^{be}	2.57 ± 0.55^{b}
	+ 25	41.56 ± 0.91^{abc}	44.19 ± 1.31^{beh}	12.63 ± 1.78^{be}	1.62 ± 0.16^{c}
Pedicel	- 1	55.64 ± 4.83^{efg}	35.37 ± 2.72^{fgh}	8.48 ± 2.47^{ade}	0.51 ± 0.20^{a}
	+ 5	60.56 ± 3.00^{gh}	33.17 ± 1.72^{f}	5.77 ± 1.12^{ac}	0.50 ± 0.19^{a}
	+ 15	56.72 ± 2.12^{eh}	36.92 ± 1.68^{fg}	5.87 ± 0.40^{ac}	0.49 ± 0.10^{a}
	+ 25	50.17 ± 1.95^{cefg}	40.19 ± 0.96^{bfg}	8.82 ± 0.90^{ce}	0.82 ± 0.22^{a}

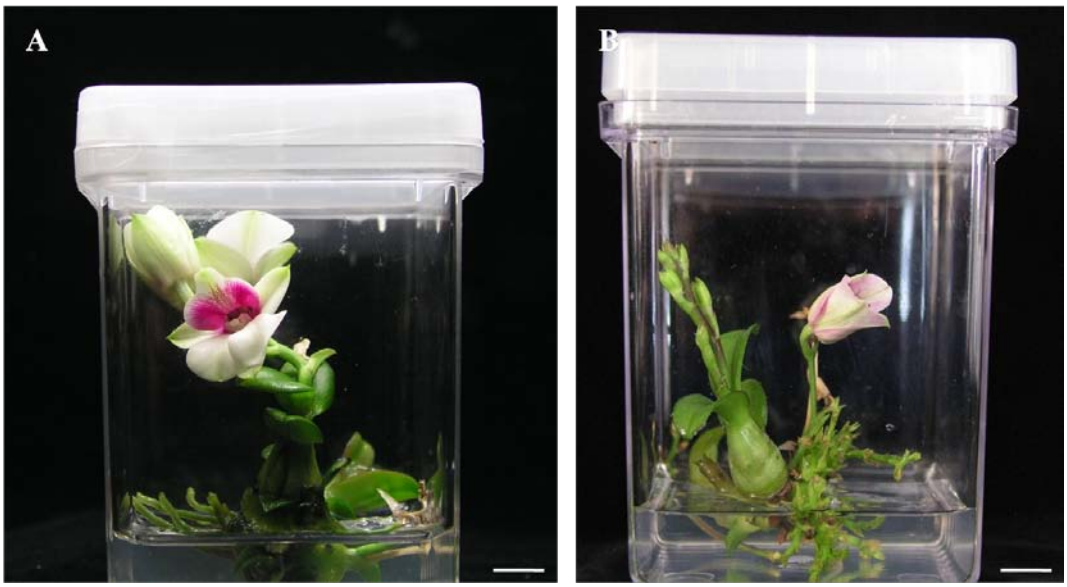

Fig 3.6. Aseptically-grown *D.* Chao Praya Smile seedlings with flowers, bar: 1.0 cm.

A: Seedling with complete flowers.

B: Seedling with deformed flower (without dorsal sepal).

Table 3.10. Mean proportion of nuclei of various DNA contents (C-values) from different floral tissues of complete flowers from aseptically-grown *D.* Chao Praya Smile seedlings during development. Different letters following the standard error within the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

Floral Part	Days After Anthesis	Mean Proportion of Nuclei (%) ± SE			
		2C	4C	8C	16C
Dorsal Sepal	5	33.41 ± 3.41^{a}	57.15 ± 1.39^{a}	8.45 ± 1.95^{ab}	0.99 ± 0.22^{ac}
	15	39.62 ± 2.64^{abde}	51.35 ± 2.30^{ac}	9.03 ± 0.36^{acd}	0.00 ± 0.00^{b}
Lateral Sepals	5	38.99 ± 4.04^{abd}	53.59 ± 2.06^{ac}	6.81 ± 2.00^{bc}	0.61 ± 0.16^{bc}
	15	45.82 ± 1.06^{abf}	47.79 ± 0.74^{abc}	6.39 ± 0.37^{bc}	0.00 ± 0.00^{b}
Petals	5	54.48 ± 8.28^{cdf}	42.17 ± 7.08^{bcd}	3.35 ± 1.21^{b}	0.00 ± 0.00^{b}
	15	56.10 ± 3.63^{cf}	40.39 ± 3.57^{bd}	3.51 ± 0.36^{bd}	0.00 ± 0.00^{b}
Labellum	5	51.65 ± 2.45^{bf}	44.13 ± 1.93^{abcd}	4.22 ± 0.57^{bd}	0.00 ± 0.00^{b}
	15	56.39 ± 1.44^{cef}	39.93 ± 1.22^{bcd}	3.68 ± 0.53^{bd}	0.00 ± 0.00^{b}
Column	5	41.80 ± 1.79^{abc}	44.13 ± 2.35^{abcd}	12.05 ± 2.04^{ac}	2.02 ± 0.43^{a}
	15	36.77 ± 2.44^{ab}	47.40 ± 2.45^{abc}	14.27 ± 2.09^{a}	1.56 ± 0.41^{ac}
Pedicel	5	60.52 ± 2.69^{f}	31.10 ± 1.19^{d}	7.54 ± 1.85^{ab}	0.84 ± 0.14^{ac}
	15	45.91 ± 1.18^{abf}	39.69 ± 1.80^{bcd}	12.27 ± 0.92^{ac}	2.13 ± 0.59^{ac}

Table 3.11. Mean proportion of nuclei of various DNA contents (C-values) from different floral tissues of deformed flowers (without dorsal sepal) from aseptically-grown *D.* Chao Praya Smile seedlings. Analysis was made 15 days after anthesis. Different letters following the standard error within the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

Floral Parts	Mean Proportion of Nuclei (%) ± SE				
	2C	4C	8C	16C	32C
Lateral Sepals	42.78 ± 4.34^{a}	50.46 ± 2.94^{a}	6.76 ± 1.97^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Labellum	47.49 ± 5.88^{a}	46.04 ± 4.26^{a}	6.47 ± 2.45^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Petals	47.76 ± 6.57^{a}	48.26 ± 6.24^{a}	3.98 ± 0.41^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Column	33.86 ± 3.07^{a}	45.16 ± 0.90^{a}	15.28 ± 1.45^{a}	5.05 ± 0.89^{b}	0.65 ± 0.23^{b}
Pedicel	46.28 ± 7.51^{a}	39.10 ± 2.56^{a}	12.82 ± 4.34^{ab}	1.81 ± 1.03^{c}	0.00 ± 0.00^{a}

3.4 Discussion

3.4.1 Occurrence of endopolyploidy in seeds and developing protocorms in culture

The seeds of *D.* Chao Praya Smile were found to contain only 2C nuclei (Table 3.1; Fig 3.5A), indicating that the cell cycle in seeds was arrested at the presynthetic G1 phase (Bino et al. 1992, Kudo and Kimura 2001a). Similar findings were described in *V.* Miss Joaquim (Lim and Loh 2003), tomato (Smulders et al. 1994), *Brassica rapa* and *B. oleracea* (Kudo and Kimura 2001a, c).

Rapid endoreduplication occurred during seed development was evident from the presence of multiploid cells with 32C nuclei after 3 weeks of culture (Table 3.1; Fig 3.5B). A further decrease in the mean frequency of 2C nuclei was observed in the 6-week-old cultures (Table 3.1; Fig 3.5C). Multiploid cells were also observed in the developing embryos of other orchids such as *V. sanderiana* (Alvarez 1968), *Cymbidium* orchids (Nagl 1972) and *V.* Miss Joaquim (Lim and Loh 2003).

Orchid seeds lacked endosperm and cells present in the embryos were heavily packed with food reserves (Arditti 1992). Developing embryos of *V. sanderiana* were reported to consist of meristemic, parenchymatous and suspensor regions (Alvarez 1968). During embryo development of *V. sanderiana*, the parenchymal cells were found to exhibit endopolyploidy. However, the meristemic cells remained 2C throughout the development (Alvarez 1968). The parenchymatous region was suggested to be analogous to the endosperm of other angiosperms, in that both tissues aided in the nutrition of the embryos (Alvarez 1968). In the development of maize endosperm, endoreduplication was required to drive the production of storage proteins (zein) and

starch which act as nutrients for the developing embryo (Lur and Setter 1993). In the parenchyma cell of cotyledons of *Pisum sativum*, an increase in endopolyploidy coincided with the rapid increase in RNA and protein synthesis (Scharpe and Van Parijs 1973). In *V.* Miss Joaquim, it was suggested that the degree of endopolyploidy aided the development of the parenchymal region to synthesise the nutrients required to support the growing protocorms (Lim and Loh 2003). Hence, the increase in the frequencies of multiploid cells in *D.* Chao Praya Smile protocorms during development could possibly served similar functions.

After 9 and 12 weeks of culture, the distribution of multiploid cells in the protocorms stabilised (Table 3.1). Similar phenomenon was observed in the protocorms of *V.* Miss Joaquim (Lim and Loh 2003). In *V. sanderiana*, it was reported that the protocorms became autotrophic when the primordial leaves and roots had developed from the meristem region. The parenchymal region of the protocorms would then slowly degrade (Alvarez 1969, 1970). In this study, protocorms with leaves (Figs 3.5D and F) were observed in the 9- and 12-week-old cultures. Therefore, after 9 weeks of culture, the nutrient needs of the protocorms might be supplemented via photosynthesis instead of endoreduplication.

3.4.2 Occurrence of systemic endopolyploidy

In *Dendrobium*, various parts of the plants had been used as explants for clonal propagation via tissue culture (Nayak et al. 1997, Ferreira et al. 2006b, Roy et al. 2007, Arditti 2008). If the explants exhibited endopolyploidy, shoots with varying ploidy levels might be regenerated (Lim and Loh 2003). Therefore, increasing the knowledge on the ploidy level of the different parts of *Dendrobium* plants would provide an insight to the nature of the explants used for micro-propagation.

Endopolyploidy in shoot tips and axillary buds

Unlike *V.* Miss Joaquim (Lim and Loh 2003), *B. rapa* and *B. oleracea* (Kudo and Kimura 2001c), polyploid cells were detected in the shoot tips and axillary buds of both greenhouse-grown plants and aseptically-grown seedlings of *D.* Chao Praya Smile (Tables 3.2 and 3.3). This indicated that in the shoot tips and axillary buds of *D.* Chao Praya Smile, some polyploid cells were intercalated among surrounding diploid cells. Endoreduplication patterns in the shoot tips and 3rd axillary buds of greenhouse-grown plants revealed that endoreduplication increased as the meristem cells in the shoot tips and axillary buds developed (Table 3.2). The 0.5-cm lateral shoots of the aseptically-grown seedlings were developed from the axillary buds. The mean frequencies of nuclei with DNA content higher than 4C in the 0.5-cm lateral shoots were found to be higher than those in the shoot tips and axillary buds of the aseptically-grown seedlings (Table 3.3). These results suggest that endopolyploidy in the shoot tips and axillary buds are developmentally regulated.

Endopolyploidy in leaves

Similar to the findings described by Jones and Kuehnle (1998), the distributions of multiploid cells in the greenhouse-grown *D.* Chao Praya Smile plants varied in different parts of the leaf (Table 3.4). The newly emerged leaves of *D.* Chao Praya Smile were light-green and unopened. They were usually concealed by the adjacent leaves (Fig 3.2B). Young leaves in the greenhouse-grown plants were found to contain multiploid cells (Table 3.4). However, in *V.* Miss Joaquim, young leaves were found to contain only 2C nuclei (Lim and Loh 2003). The endoreduplication process was observed to be most rapid and active in newly emerged leaves as indicated by the difference in the distribution of multiploid cells in the 1st and 2nd leaves of the

greenhouse-grown *D.* Chao Praya Smile plants (Table 3.4). As the leaves developed, there was a gradual shift in the ploidy distributions towards higher ploidy levels and new rounds of endoreduplication were observed to start from the leaf tip in a basipetal manner as indicated in the 4th leaf (Table 3.4). However, the difference in the ploidy variations between the different parts of the leaves stabilised once they matured (Table 3.4). This is consistent with the findings previously described in *V.* Miss Joaquim (Lim and Loh 2003) and *S. plicata* (Yang and Loh 2004). The increase in the occurrence of multiploid cells in a basipetal manner in the leaf tissues suggests that endoreduplication in the greenhouse-grown *D.* Chao Praya Smile plants is developmentally regulated. In *Arabidopsis,* endoreduplication in the leaves was also found to be developmentally regulated (Galbraith et al. 1991). In the leaf epidermis cells of *Arabidopsis,* it was found that cell cycle genes such as CDT1 and CDC6 exhibited a positive role in the regulation of endoreduplication (Castellano et al. 2001, Castellano et al. 2004). Castellano et al. (2004) showed that in cells that were competent to divide, an increase in CDT1 and CDC6 levels would result in cell proliferation, while in cells programmed to undergo endoreduplication, extra rounds of endoreduplication cycle would be triggered. These genes were also important for the coordination of cell proliferation, differentiation and development (Castellano et al. 2001, Castellano et al. 2004). Therefore, in orchids, similar regulatory mechanism could possibly be involved in the regulation of endoreduplication during development. However, further verification is required to validate this speculation.

Analyses of the first leaf from plants just before bolting and 4 months after bolting further demonstrated that once the leaves matured, the distribution of multiploid cells between the tip and the basal sections of the same leaf did not differ significantly (Table 3.5). However, further increase in endopolyploidy in the whole leaves was

observed as the leaves aged (Table 3.5). This indicated that endoreduplication might be closely associated with aging. There were evidence in other plant species that endoreduplication and aging/senescing were linked. For instance, in tomato, significantly more nuclei with higher DNA content were detected in the leaf tips of yellowing leaves (senescing) (Smulders et al. 1994). In the maize endosperm, the aleurone (outermost layer of cells) was reported to be analogous to the meristem by providing more cells to the inner regions of the kernel. In the inner regions, endoreduplication of the cells was observed to increase during development. Endoreduplication of the central region of maize endosperm was reported to be related to the endosperm development (Kowles and Phillips 1985, Kowles et al. 1990). In the central endosperm of maize, the completion of endoreduplication was followed by rapid cell death, suggesting that these two processes were linked (Young and Gallie 2000). In maize and tobacco, homologues of the retinoblastoma protein, which was involved in cell cycle control, cell differentiation and apoptosis in animals, have been identified (Grafi et al. 1996, Nakagami et al. 2002). These homologues were suggested to be involved in the regulation of endoreduplication (Park et al. 2005), supporting the relationship between aging/cell death and endoreduplication.

The older leaves of aseptically-grown seedlings of *D.* Chao Praya Smile also exhibited higher ploidy level than younger leaves and the ploidy level increased in a basipetal manner (Table 3.6). However, fully expanded leaves of the aseptically-grown seedlings were found to have lower level of endopolyploidy than that of the greenhouse-grown plants (Tables 3.4 and 3.6). In the oldest leaf (5^{th} leaf) of the aseptically-grown seedlings, the mean frequency of 2C nuclei was 34.86 % (Table 3.6). However, in the greenhouse-grown plants, only a mean frequency of 15.32 % of the nuclei in the oldest leaves (6^{th} leaf) was found to contain 2C DNA content (Table

3.4). This is consistent with Smulders et al. (1994) who reported that the ploidy levels of the cotyledons and leaves of greenhouse-grown tomato plants were much higher as compared to the aseptically-grown tomato seedlings. The mean frequency of 2C nuclei in the cotyledons of aseptically-grown tomato seedlings was 51.9 %, while that of the greenhouse-grown tomato plants was only 9.5 % (Smulders et al. 1994). Smulders et al. (1994) suggested that the growth conditions such as light would influence the degree of endopolyploidy in plants. In the current study, greenhouse-grown *D.* Chao Praya Smile plants were exposed to natural lighting of intensity about 90.4 µE m^{-2} s^{-1}, while aseptically-grown seedlings were maintained under illumination of intensity about 45.9 µE m^{-2} s^{-1}. This implies that the difference in the extent of endopolyploidy in the greenhouse-grown plants and aseptically-grown seedlings of *D.* Chao Praya Smile could be due the difference in the light intensity. Moreover, studies have shown that light would affect the extent of endoreduplication in plants. These studies demonstrated that the ploidy levels of dark-grown seedlings such as *Triticum durum* (Cavallini et al.1995), *P. sativum* (Callebaut et al. 1982), *Glycine max* (Galli 1988) and *Arabidopsis* (Tsumoto et al. 2006, Gendreau et al. 1998) were higher than those seedlings grown under the light.

On the other hand, in potatoes, only small differences were observed in the ploidy levels between the greenhouse-grown plants and aseptically-grown seedlings. The difference between the mean frequencies of 2C nuclei in these two types of plant was only 6 % (Uijtewaal 1987). Furthermore, the endopolyploidy patterns in *S. plicata* were found to be similar in both aseptically-grown seedlings and greenhouse-grown plants (Yang and Loh 2004). These demonstrated that the effects of growth conditions on endopolyploidy varied with plants.

Endopolyploidy in roots

The roots of *D.* Chao Praya Smile were divided into parts to determine the actual localisation of the polyploid cells. The results revealed that endoreduplication was not restricted to specific regions of the roots in both greenhouse-grown plants and aseptically-grown seedlings (Tables 3.7 and 3.8). Endopolyploidy patterns appeared to be coupled with development. Jones and Kuehnle (1998) reported that multiploid cells were observed in the root tips of four *Dendrobium* species and nuclei with up to 8C DNA content were observed. The ploidy variations in the root tips of these *Dendrobium* species were observed to differ greatly with their mean frequencies of 8C nuclei ranging from 0 to 41.7 %. In the root tips of greenhouse-grown *D.* Chao Praya Smile plants, only 9.27 % of the total nuclei population analysed was found to contain 8C DNA content (Table 3.7). Orchid roots consisted of different cell layers namely endodermis, exodermis, stele and velamen (Arditti 1992). The roots of epiphytic orchids, such as *Dendrobium* plants, were observed to vary in habits, appearance, and cross-section among and within genera and species (Arditti 1992). These differences could have contributed to the variations in the endopolyploidy patterns among these *Dendrobium* plants. In general, the roots from aseptically-grown *D.* Chao Praya Smile seedlings were found to exhibit higher ploidy level as compared to that from the greenhouse-grown plants (Tables 3.7 and 3.8). This could possibly be due to the medium compositions as the root systems of aseptically-grown *D.* Chao Praya Smile seedlings were submerged in nutrient-rich medium. In the current study, coconut water was one of the medium components. It was reported to contain a large spectrum of uncharacterised biochemicals (Ma et al. 2008, Shantz and Steward 1952). For instance, biochemicals such as 1,3-diphenylurea (which exhibits cytokinin-like activity) (Shantz and Steward 1955), cytokinins (Van Staden and Drewes 1975, Ge et al. 2004, 2005),

indole-3-acetic acid (IAA) and abscisic acid (Ma et al. 2008) were detected in the coconut water. These biochemicals could act as plant growth regulators individually or synergistically (Ma et al. 2008, Shantz and Steward 1952). Plant growth regulators such as auxins and cytokinins could affect the endopolyploidy patterns of orchids (Mishiba et al. 2001, Lim and Loh 2003), tobacco (Valente et al. 1998), bean (Kinoshita et al. 1991) and pea (Libbenga and Torrey 1973). Therefore, the presence of these growth regulators in coconut water may affect the degree of endopolyploidy in the roots of aseptically-grown *D.* Chao Praya Smile seedlings cultured in medium supplemented with coconut water.

Endopolyploidy in flowers

Multiploid cells were found in the flowers of greenhouse-grown plants (Table 3.9) and complete flowers of aseptically-grown seedlings of *D.* Chao Praya Smile (Table 3.10). In the flowers of *B. oleracea* (Kudo and Kimura 2001b) and *Portulaca grandiflora* (Mishiba and Mii 2000), multiploid cells were also detected. However, in the flowers of *D.* Chao Praya Smile plants, no significant change in the ploidy variations in all the floral tissues examined after anthesis was observed (Tables 3.9 and 3.10). This indicated that endoreduplication in the floral tissues was not developmentally regulated. In the flowers of other orchids such as *Phalaenopsis* and *Oncidium*, endoreduplication stopped and ploidy level stabilised once the flowers were fully opened (Lee et al. 2004). Thus, endoreduplication in the floral tissues of *D.* Chao Praya Smile might have ceased before anthesis.

The ploidy variation in the column of the flowers of *D.* Chao Praya Smile was different from other floral parts; the columns contained higher mean frequencies of nuclei with 8C DNA content (12.18 to 14.11 %) and 16C DNA content (1.62 to 2.57

%) (Table 3.9). Similarly, the ploidy levels in the columns of *V.* Miss Joaquim flowers were found to be higher than other floral parts (Lim and Loh 2003). Since the column of orchid flower is a modified organ derived from the fusion of stigma, style and anthers (Hew and Yong 2004), it was suggested that the unique endopolyploidy patterns in the column could be due to its structural modification (Lim and Loh 2003). However, a detail histological and cytological analysis on the column of *D.* Chao Praya Smile flowers would be required to provide further insight on the effect of this structural modification on endopolyploidy in the *D.* Chao Praya Smile flowers.

Endoreduplication has been reported to be common in the reproductive organs and tissues of angiosperms (D'Amato 1984) and in specific cell types or tissues (Nagl 1976, Galbraith et al. 1991, Carvalheira 2000) such as endosperms of maize (Kowles and Phillips 1985) and raphide crystal idioblasts of *Vanilla* (Kausch and Horner 1984). Galbraith et al. (1991) suggested that endoreduplication was common in cells that were required to increase their size in order to maintain their functions. It was proposed that in these tissues, endoreduplication was required to increase the transcriptional and metabolic activities which were necessary for their functions (D'Amato 1984). For instance, in the parenchyma cell of cotyledons of *P. sativum*, the increase in polyploidy coincided with a rapid increase in RNA and protein synthesis (Scharpe and Van Parijs 1973). In sugar-beet plant, high degree of endopolyploidy was observed in the storage parenchyma cells of roots (Lukaszewska and Sliwinska 2007). Therefore, the higher degree of endopolyploidy in the column of *D.* Chao Praya Smile could possibly serve to increase its RNA and protein synthesis so as to maintain its function as a reproductive organ. However, further verification is required to validate this speculation.

From the aseptically-grown seedlings, some of the flowers induced were deformed (Fig 3.6B). In such flowers, the ploidy levels in the column were higher than that of the flowers from greenhouse-grown plants and the complete flowers from aseptically-grown seedlings (Tables 3.9, 3.10 and 3.11). This suggests that deformity in flowers could possibly result in an increase in endopolyploidy. Lim and Loh (2003) reported that in the column of complete flower from greenhouse-grown *V.* Miss Joaquim plants, cell types of different sizes and nuclear DNA contents were present. Similar phenomenon could possibly exist in the columns of *D*. Chao Praya Smile flowers. The deformity in the flowers from aseptically-grown seedlings could have resulted in changes in the cell types and nuclear DNA contents in the column of the deformed flowers. Therefore, to determine the cause of the increase in ploidy level in the deformed flowers, confocal microscopic and cytological studies on the columns of the deformed and complete flowers are required.

In this chapter, detailed analyses of endopolyploidy in greenhouse-grown plants and aseptically-grown *D.* Chao Praya Smile seedlings revealed the presence of systemic control of endopolyploidy. Multiploid cells were found in majority of the tissues analysed. In most of the tissues analysed, endopolyploidy was observed to be developmentally regulated. In addition, the extent of endopolyploidy in the roots and leaves of aseptically-grown seedlings was different from that of greenhouse-grown plants, suggesting that plant growth conditions such as light and medium compositions could have an effect on endopolyploidy in *D.* Chao Praya Smile.

Chapter 4 Effects of plant growth regulators on endopolyploidy in the protocorm cultures of *D.* Chao Praya Smile

4.1 Introduction

During micro-propagation of orchids, plant growth regulators such as cytokinins and auxins have often been used (Nayak et al. 1997, 2002, Arditti 2008). For instance, 2,4-D has been used in callus induction from the root tip explants of *Cattleya* (Kerbauy 1991). Auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) induced somatic embryogenesis from the leaf explants of *Oncidium* (Chen and Chang 2005). Cytokinin such as BA has been used to induce flowers from aseptically-grown *Cymbidium* (Kostenyuk et al. 1999) and *Dendrobium* seedlings (Sim et al. 2007, Hee et al. 2007). In *Cymbidium*, GA was found to delay flowering, while paclobutrazol (PAC, GA biosynthesis inhibitor) blocked the inductive effect of cytokinin on flowering (Kostenyuk et al. 1999).

The addition of plant growth regulators to the culture medium could affect the extent of somaclonal variation in the regenerants by affecting cell cycle or endoreduplication cycle in the explants (Brar and Jain 1998). Previous studies showed that plant growth regulators such as cytokinins, auxins and GAs would either enhance, reduce or have no effect on endoreduplication in aseptically-grown plantlets (Kinoshita et al. 1991, Gendreau et al. 1999), callus cultures (Libbenga and Torrey 1973), suspension cell cultures (Valente et al. 1998, Mishiba et al. 2001) and protocorm cultures (Lim and Loh 2003). The effect of GA on endopolyploidy appeared to be dosage (Lim and Loh 2003) and cultivar (Callebaut et al. 1982, Cavallini et al. 1995) dependent.

In the orchid *Doritaenopsis*, addition of cytokinin to the culture medium was reported to result in a slight decrease in endoreduplication in the suspension cell cultures (Mishiba et al. 2001). However, the effects of auxins on endoreduplication in the suspension cells of *Doritaenopsis* varied, depending on the types of auxin used. In the culture medium supplemented with 2,4-D or picloram, an increase in endoreduplication was observed. Yet in media containing other types of auxin, no effect on endopolyploidy in the suspension cells was observed (Mishiba et al. 2001). In the protocorms of *V.* Miss Joaquim, the addition of 10.0 µM α-naphthaleneacetic acid (NAA) to the culture medium enhanced endoreduplication (Lim and Loh 2003). In culture medium supplemented with 10.0 µM GA_3, only slight increase in endoreduplication was observed (Lim and Loh 2003). The effects of these plant growth regulators on endopolyploidy in *Dendrobium* have not been reported.

Hence, the objective of this chapter is to study the effects of a common cytokinin, BA, auxin, 2,4-D, and a gibberellic acid, GA_3, on endopolyploidy in the protocorm cultures of *D.* Chao Praya Smile. In addition, the effects of an auxin transport inhibitor, TIBA, and PAC, a GA biosynthesis inhibitor, were also studied.

4.2 Materials and methods

4.2.1 Plant materials

D. Chao Praya Smile (*D.* Pinky × *D.* Kiyomi Beauty) plants were grown in pots in the greenhouse in the Department of Biological Sciences, National University of Singapore, at 28 ± 4 ºC with natural lighting of intensity 90.4 ± 12.4 µE m^{-2} s^{-1}. Flowers from the greenhouse-grown plants were hand-pollinated.

Three-month-old seed pods were washed with detergent, surface sterilised in 20 % (v/v) Clorox^{TM} solution with a drop of Tween 20 for 15 min with constant agitation. The seed pods were then rinsed twice with autoclaved water and cut into halves. Seeds were germinated aseptically in 50 ml of basal KC medium (KC, Knudson 1946) supplemented with 2 % (w/v) sucrose and 15 % (v/v) coconut water at pH 5.3 in 150 ml Erlenmeyer flasks on rotary shakers at 120 rpm. The cultures were maintained at 25 ºC under 16 hours illumination of intensity 45.9 ± 1.3 µE m^{-2} s^{-1}.

4.2.2 Effects of plant growth regulators on endopolyploidy

Three-week-old protocorms were transferred to 50 ml of KC medium supplemented with BA, 2,4-D, GA_3, TIBA, absolute ethanol or PAC. All cultures were maintained on a rotary shaker at 120 rpm at 25 ºC under 16 hours illumination of intensity 45.9 ± 1.3 µE m^{-2} s^{-1} and subculturing was done every 3 weeks. Flow cytometric analyses of protocorms were conducted every 3 weeks for a period of 6 weeks.

4.2.3 Nuclei isolation for flow cytometric analyses

Extraction of nuclei was conducted according to section 3.2.2.

4.2.4 Flow cytometric analyses

Method described in section 3.2.3 was used for flow cytometric analyses.

4.2.5 Statistical analyses

All percentage data were analysed with one-way analysis of variance (ANOVA) and Tukey's test at 95 % confidence level was performed for multiple pair-wise comparisons. Data were arcsine-transformed to meet assumptions of ANOVA (normality and homogeneity of variance) and analysed with Minitab™ 15.

4.3 Results

4.3.1 Effect of BA

Compared to protocorms cultured in basal KC medium for 3 weeks, a significant decrease in endopolyploidy was observed in the protocorms cultured in media containing BA (Table 4.1; Figs 4.1A - D). For protocorms cultured in basal KC medium (Fig 4.1A), the mean frequency of 2C nuclei was 46.61 %, which was significantly lower than that in the protocorms cultured in medium containing 0.1 µM BA (48.82 %) (Table 4.1). However, the mean frequencies of nuclei with DNA content higher than 2C from the protocorms cultured in medium with 0.1 µM BA (Fig 4.1B) did not differ significantly from those of the protocorms cultured in basal KC medium (Table 4.1).

Protocorms cultured in medium supplemented with 10.0 µM BA (Fig 4.1D) contained a mean frequency of 56.88 % 2C nuclei, which was significantly higher than that in the protocorms cultured in basal KC medium and medium containing 0.1 µM BA (Table 4.1). Conversely, the mean frequencies of 8C and 16C nuclei in the protocorms cultured in medium supplemented with 10.0 µM BA were significantly lower as compared to the protocorms cultured in basal KC medium and medium containing 0.1 µM BA (Table 4.1). 32C and 64C nuclei were not detected in protocorms cultured in medium with 10.0 µM BA for 3 weeks (Table 4.1).

After 6 weeks of culture, some protocorms cultured in basal KC medium and media containing 0.1 and 1.0 µM BA developed leaves (Figs 4.2A, C and E). However, majority of the protocorms remained leafless (Figs 4.2B, D and F). For protocorms

cultured in medium supplemented with 10.0 µM BA, only leafless protocorms were observed (Fig 4.2G).

For protocorms cultured in medium containing 10.0 µM BA for 6 weeks, the mean frequency of 2C nuclei was 50.37 %. This was significantly higher than the mean frequency of 2C nuclei in the leafless protocorms cultured in basal KC medium (31.82 %) and media with 0.1 and 1.0 µM BA for 6 weeks (33.18 and 36.48 %, respectively) (Table 4.1).

In medium containing 10.0 µM BA for 6 weeks, 32C nuclei were detected and less 2C nuclei (about 6 % less) were found in the protocorms cultured compared to those cultured in the same medium for 3 weeks (Table 4.1; Figs 4.1D and 4.2G).

Table 4.1. Effect of BA on the pattern of endopolyploidy in the protocorms of *D.* Chao Praya Smile cultured in liquid basal KC media supplemented with BA, based on flow cytometric analyses of 10 000 nuclei per replicate. Within the same week of culture, different letters following the standard error for the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

BA (µM)	Type of Culture	Mean Proportion of Nuclei (%) ± SE					
		2C	4C	8C	16C	32C	64C
3 Weeks After Culture							
0.0	Protocorms	46.61 ± 0.30^{a}	31.59 ± 0.32^{a}	13.76 ± 0.28^{a}	5.73 ± 0.39^{a}	1.84 ± 0.13^{ac}	0.47 ± 0.17^{a}
0.1	Protocorms	48.82 ± 0.21^{b}	31.63 ± 0.39^{a}	12.02 ± 0.21^{a}	4.99 ± 0.23^{ab}	1.92 ± 0.09^{a}	0.62 ± 0.07^{a}
1.0	Protocorms	50.39 ± 0.34^{c}	32.16 ± 0.30^{a}	11.68 ± 0.22^{ab}	4.20 ± 0.12^{b}	1.57 ± 0.05^{c}	0.00 ± 0.00^{b}
10.0	Protocorms	56.88 ± 0.32^{d}	30.68 ± 1.00^{a}	9.71 ± 0.85^{b}	2.73 ± 0.22^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
6 Weeks After Culture							
0.0	Protocorms with Leaves	43.17 ± 0.21^{ac}	32.55 ± 1.00^{a}	15.12 ± 0.77^{ab}	7.34 ± 0.21^{bc}	1.82 ± 0.45^{a}	0.00 ± 0.00^{a}
	Protocorms	31.82 ± 0.12^{d}	34.68 ± 0.81^{a}	19.57 ± 1.03^{c}	11.08 ± 0.74^{bc}	2.85 ± 0.58^{a}	0.00 ± 0.00^{a}
0.1	Protocorms with Leaves	43.42 ± 2.20^{ac}	33.20 ± 1.03^{a}	15.17 ± 0.93^{ab}	6.35 ± 0.75^{ac}	1.86 ± 0.63^{a}	0.00 ± 0.00^{a}
	Protocorms	33.18 ± 0.59^{bd}	31.80 ± 1.23^{a}	20.60 ± 0.40^{c}	11.80 ± 0.88^{b}	2.62 ± 0.84^{a}	0.00 ± 0.00^{a}
1.0	Protocorms with Leaves	39.39 ± 0.91^{bc}	34.46 ± 0.74^{a}	16.38 ± 1.09^{abc}	8.27 ± 0.70^{bc}	1.53 ± 0.34^{a}	0.00 ± 0.00^{a}
	Protocorms	36.48 ± 3.33^{bcd}	34.42 ± 0.60^{a}	18.23 ± 1.15^{bc}	8.33 ± 2.27^{bc}	2.54 ± 0.98^{a}	0.00 ± 0.00^{a}
10.0	Protocorms	50.37 ± 0.78^{a}	32.26 ± 0.77^{a}	13.12 ± 0.67^{a}	3.29 ± 0.13^{a}	0.96 ± 0.05^{a}	0.00 ± 0.00^{a}

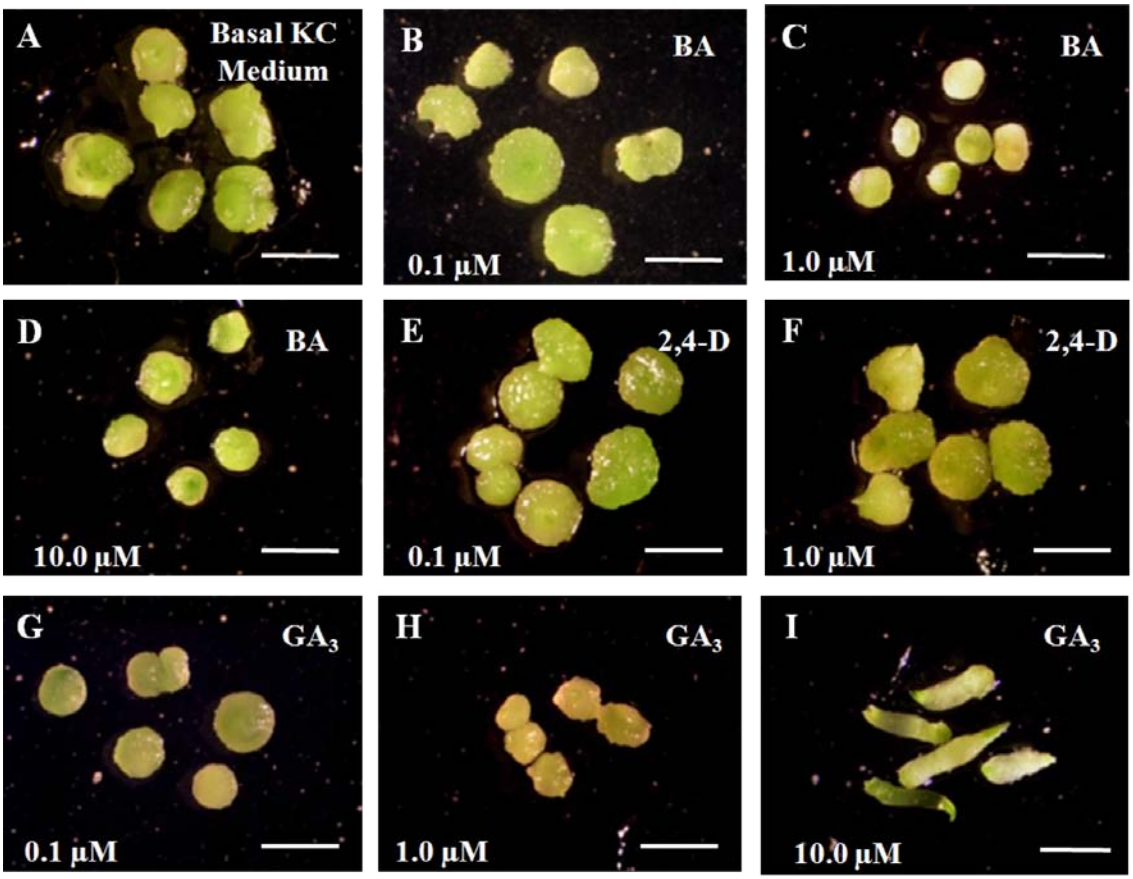

Fig 4.1. Protocorms of *D.* Chao Praya Smile cultured for 3 weeks in liquid basal KC medium or media supplemented with BA, 2,4-D or GA_3, bar: 0.3 cm.

A: Basal KC medium.

B - D: Basal KC media with BA.

E , F: Basal KC media with 2,4-D.

G - I: Basal KC media with GA_3.

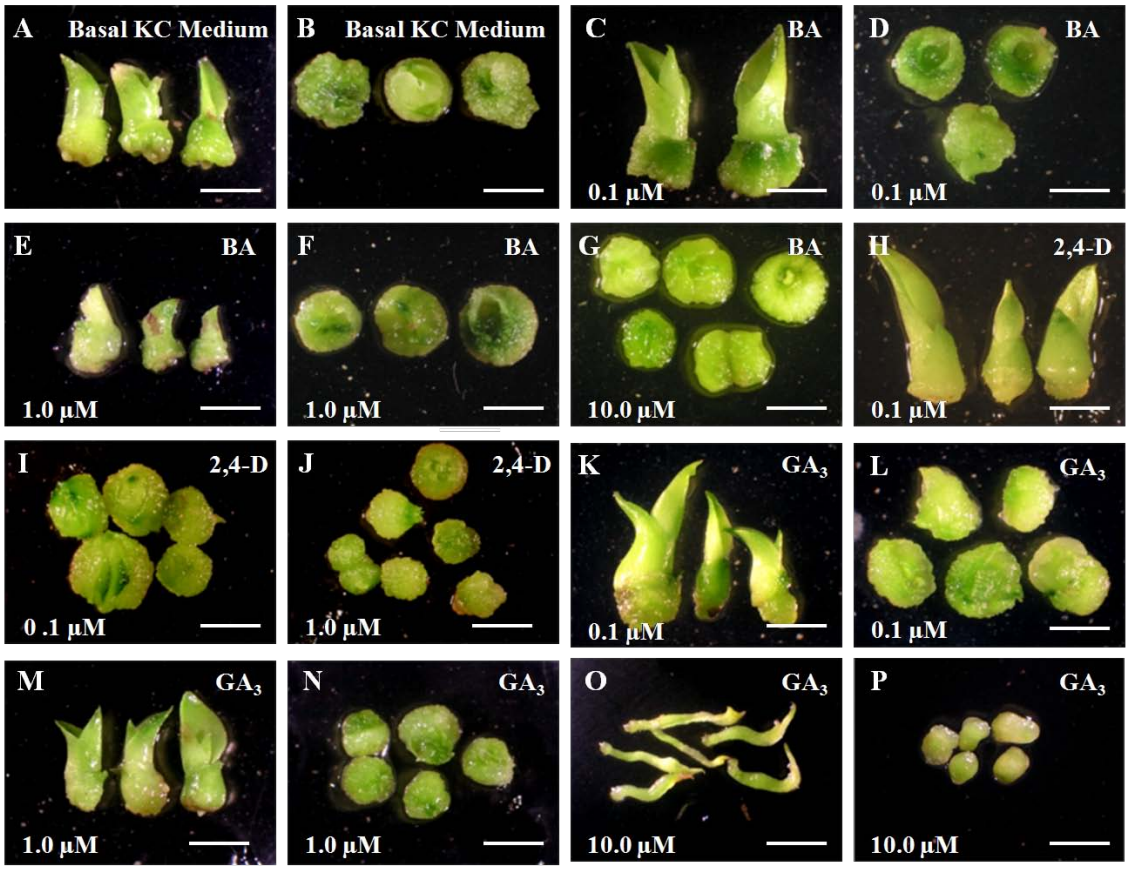

Fig 4.2. Protocorms of *D.* Chao Praya Smile cultured for 6 weeks in liquid basal KC medium or media supplemented with BA, 2,4-D or GA_3.

A , B: Basal KC medium (**A**: Protocorms with leaves), bar: 0.41 cm.

C - G: Basal KC media with BA (**C**, **E**: Protocorms with leaves), bar: 0.53 cm.

H - J: Basal KC media with 2,4-D (**H**: Protocorms with leaves), bar: 0.42 cm.

K - P: Basal KC media with GA_3 (**K**, **M**: Protocorms with leaves, **O**: Elongated protocorms), bar: 0.30 cm.

4.3.2 Effect of 2,4-D

Protocorms cultured in medium with 10.0 µM 2,4-D for 3 weeks turned brown and could not be used for nuclei isolation. Thus, only protocorms cultured in media containing 0.1 and 1.0 µM 2,4-D were analysed. Protocorms cultured in basal KC medium for 3 weeks (Fig 4.1A) contained a mean frequency of 46.61 % nuclei with 2C DNA content. This was significantly higher than the mean frequency of 2C nuclei in the protocorms cultured in media containing 0.1 and 1.0 µM 2,4-D (44.77 and 40.79 %, respectively) for 3 weeks (Table 4.2; Figs 4.1E and F). In medium containing 1.0 µM 2,4-D, a significant increase was observed in the mean frequencies of 8C, 16C, 32C and 64C nuclei in the protocorms cultured as compared to those cultured in basal KC medium and medium supplemented with 0.1 µM 2,4-D (Table 4.2).

After 6 weeks of culturing in medium containing 0.1 µM 2,4-D, some protocorms developed leaves (Fig 4.2H), but majority of the protocorms remained leafless (Fig 4.2I). In medium containing 1.0 µM 2,4-D, only leafless protocorms were observed (Fig 4.2J) and they contained 64C nuclei with a mean frequency of 1.17 % (Table 4.2). However, in basal KC medium and medium containing 0.1 µM 2,4-D for 6 weeks, the highest DNA content detected in the protocorms was 32C (Table 4.2).

Table 4.2. Effect of 2,4-D on the pattern of endopolyploidy in the protocorms of *D.* Chao Praya Smile cultured in liquid basal KC media supplemented with 2,4-D, based on flow cytometric analyses of 10 000 nuclei per replicate. Within the same week of culture, different letters following the standard error for the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

2,4-D (µM)	Type of Culture	Mean Proportion of Nuclei (%) ± SE					
		2C	4C	8C	16C	32C	64C
3 Weeks After Culture							
0.0	Protocorms	46.61 ± 0.30^{a}	31.59 ± 0.32^{a}	13.76 ± 0.28^{a}	5.73 ± 0.39^{a}	1.84 ± 0.13^{a}	0.47 ± 0.17^{a}
0.1	Protocorms	44.77 ± 0.27^{b}	32.67 ± 0.60^{a}	13.31 ± 0.17^{a}	6.03 ± 0.38^{a}	2.42 ± 0.15^{a}	0.80 ± 0.13^{a}
1.0	Protocorms	40.79 ± 0.23^{c}	31.20 ± 0.52^{a}	15.69 ± 0.13^{b}	7.71 ± 0.25^{b}	3.50 ± 0.29^{b}	1.11 ± 0.09^{b}
6 Weeks After Culture							
0.0	Protocorms with Leaves	43.17 ± 0.21^{a}	32.55 ± 1.00^{a}	15.12 ± 0.77^{a}	7.34 ± 0.21^{a}	1.82 ± 0.45^{a}	0.00 ± 0.00^{a}
	Protocorms	31.82 ± 0.12^{b}	34.68 ± 0.81^{a}	19.57 ± 1.03^{b}	11.08 ± 0.74^{ab}	2.85 ± 0.58^{ab}	0.00 ± 0.00^{a}
0.1	Protocorms with Leaves	41.02 ± 1.10^{a}	32.51 ± 0.65^{a}	16.74 ± 1.22^{ab}	7.36 ± 0.64^{a}	2.37 ± 0.51^{ab}	0.00 ± 0.00^{a}
	Protocorms	33.90 ± 0.26^{b}	31.98 ± 2.53^{a}	19.57 ± 0.60^{b}	11.25 ± 1.56^{b}	3.30 ± 0.67^{ab}	0.00 ± 0.00^{a}
1.0	Protocorms	32.80 ± 0.76^{b}	31.02 ± 0.92^{a}	19.04 ± 0.76^{b}	11.41 ± 0.89^{b}	4.56 ± 0.13^{b}	1.17 ± 0.16^{b}

4.3.3 Effect of GA_3

Compared to protocorms cultured in basal KC medium for 3 weeks, the presence of GA_3 in the culture medium resulted in a significant decrease in the mean frequency of 2C nuclei and an increase in 4C nuclei in the protocorms (Table 4.3; Figs 4.1A and G - I). The addition of 10.0 µM GA_3 to the culture medium resulted in about 2 % decrease in the mean frequency of 2C nuclei and about 4 % increase in 4C nuclei in the protocorms as compared to those cultured in basal KC medium (Table 4.3).

After culturing in media containing 0.1 and 1.0 µM of GA_3 for 6 weeks, majority of the protocorms remained leafless (Figs 4.2L and N). However, a small population of the protocorms was observed to have developed leaves (Figs 4.2K and M). In medium containing 10.0 µM GA_3 for 6 weeks, the protocorms remained leafless and majority of the protocorms were elongated (Figs 4.2O and P). The elongated protocorms cultured in medium supplemented with 10.0 µM GA_3 for 6 weeks contained less 2C nuclei (about 11 % less) and more 8C nuclei (about 6 % more) than the protocorms with leaves in the basal KC medium (Table 4.3; Figs 4.2A and O). In the medium containing 10.0 µM of GA_3 for 6 weeks, the elongated protocorms contained significantly higher mean frequency of 16C nuclei (10.47 %) than that in the non-elongated protocorms (6.06 %) (Table 4.3; Figs 4.2O and P).

Table 4.3. Effect of GA_3 on the pattern of endopolyploidy in the protocorms of *D.* Chao Praya Smile cultured in liquid basal KC media supplemented with GA_3, based on flow cytometric analyses of 10 000 nuclei per replicate. Within the same week of culture, different letters following the standard error for the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

GA_3 (µM)	Type of Culture	Mean Proportion of Nuclei (%) ± SE					
		2C	4C	8C	16C	32C	64C
3 Weeks After Culture							
0.0	Protocorms	46.61 ± 0.30^{a}	31.59 ± 0.32^{a}	13.76 ± 0.28^{a}	5.73 ± 0.39^{a}	1.84 ± 0.13^{a}	0.47 ± 0.17^{a}
0.1	Protocorms	43.95 ± 0.75^{b}	34.02 ± 0.53^{b}	14.34 ± 0.22^{ab}	5.60 ± 0.11^{a}	1.63 ± 0.06^{ac}	0.46 ± 0.15^{a}
1.0	Protocorms	43.61 ± 0.26^{b}	33.54 ± 0.48^{b}	15.15 ± 0.16^{b}	5.91 ± 0.14^{a}	1.26 ± 0.11^{bc}	0.53 ± 0.23^{a}
10.0	Protocorms	44.16 ± 0.40^{b}	35.90 ± 0.23^{c}	14.62 ± 0.16^{ab}	4.29 ± 0.14^{b}	1.03 ± 0.10^{b}	0.00 ± 0.00^{a}
6 Weeks After Culture							
0.0	Protocorms with Leaves	43.17 ± 0.21^{a}	32.55 ± 1.00^{ab}	15.12 ± 0.77^{a}	7.34 ± 0.21^{ac}	1.82 ± 0.45^{ac}	0.00 ± 0.00^{a}
	Protocorms	31.82 ± 0.12^{bc}	34.68 ± 0.81^{ac}	19.57 ± 1.03^{b}	11.08 ± 0.74^{bcd}	2.85 ± 0.58^{ab}	0.00 ± 0.00^{a}
0.1	Protocorms with Leaves	34.77 ± 4.22^{ab}	31.53 ± 0.55^{bc}	20.44 ± 2.03^{b}	10.32 ± 1.57^{bc}	2.94 ± 0.61^{ab}	0.00 ± 0.00^{a}
	Protocorms	28.97 ± 2.70^{b}	29.30 ± 0.38^{b}	21.68 ± 1.14^{b}	15.63 ± 1.22^{d}	4.42 ± 0.29^{b}	0.00 ± 0.00^{a}
1.0	Protocorms with Leaves	41.30 ± 1.74^{ac}	31.49 ± 1.11^{bc}	16.88 ± 0.59^{ab}	7.57 ± 0.45^{ac}	2.76 ± 0.33^{ab}	0.00 ± 0.00^{a}
	Protocorms	29.36 ± 1.92^{b}	31.09 ± 1.41^{bc}	22.12 ± 1.14^{b}	13.44±1.49^{bd}	3.99 ± 0.84^{bc}	0.00 ± 0.00^{a}
10.0	Elongated Protocorms	32.39 ± 2.29^{bc}	33.93 ± 1.33^{ab}	21.01 ± 0.94^{b}	10.47 ± 0.73^{bc}	2.20 ± 0.44^{ab}	0.00 ± 0.00^{a}
	Protocorms	38.38 ± 1.03^{ab}	36.25 ± 0.92^{a}	18.10 ± 0.89^{ab}	6.06 ± 0.41^{a}	1.21 ± 0.07^{a}	0.00 ± 0.00^{a}

4.3.4 Effect of TIBA

Compared to protocorms cultured in basal KC medium for 3 weeks, no significant difference was observed in the mean frequencies of 2C, 8C and 64C nuclei in the protocorms cultured in medium supplemented with 50.0 μM TIBA (Table 4.4; Figs 4.3A and C). However, the presence of 50.0 μM TIBA in the culture medium resulted in about 1 % increase in the mean frequencies of 16C and 32C nuclei in the protocorms after 3 weeks as compared to protocorms cultured in basal KC medium (Table 4.4). Protocorms cultured in medium containing 1.0 μM 2,4-D had significantly less 2C nuclei than those cultured in basal KC medium and medium containing 50.0 μM TIBA (Table 4.4; Fig 4.3). Conversely, the protocorms in medium containing 1.0 μM 2,4-D had more 8C, 16C and 64C nuclei as compared to those cultured in basal KC medium and medium with 50.0 μM TIBA (Table 4.4).

After culturing in medium containing 50.0 μM TIBA for 6 weeks, the mean frequency of 2C nuclei in the protocorms (43.09 %) was significantly higher as compared to the protocorms cultured in basal KC medium (33.29 %) and medium containing 1.0 μM 2,4-D (35.06 %) (Table 4.4; Fig 4.4).

On the other hand, no difference in the ploidy variation was observed in the protocorms cultured in medium containing 50.0 μM TIBA for 3 and 6 weeks (Table 4.4).

Table 4.4. Effects of 2,4-D and TIBA on the pattern of endopolyploidy in the protocorms of *D.* Chao Praya Smile cultured in liquid basal KC medium and KC medium supplemented with 1.0 µM 2,4-D or 50.0 µM TIBA, based on flow cytometric analyses of 10 000 nuclei per replicate. Within the same week of culture, different letters following the standard error for the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

Plant Growth Regulator	Type of Culture	Mean Proportion of Nuclei (%) ± SE					
		2C	4C	8C	16C	32C	64C
3 Weeks After Culture							
-	Protocorms	43.30 ± 0.89^{a}	33.76 ± 0.25^{a}	13.85 ± 0.35^{a}	5.92 ± 0.21^{a}	2.32 ± 0.11^{a}	0.85 ± 0.15^{a}
2,4-D	Protocorms	40.08 ± 0.84^{b}	31.74 ± 0.32^{b}	15.98 ± 0.29^{b}	7.34 ± 0.12^{b}	3.55 ± 0.15^{b}	1.31 ± 0.06^{b}
TIBA	Protocorms	45.43 ± 0.40^{a}	29.66 ± 0.50^{c}	14.26 ± 0.17^{a}	6.51 ± 0.05^{c}	3.40 ± 0.08^{b}	0.74 ± 0.04^{a}
6 Weeks After Culture							
-	Protocorms with Leaves	41.88 ± 1.04^{ac}	30.95 ± 0.37^{a}	16.84 ± 1.47^{ab}	7.71 ± 0.21^{a}	2.62 ± 0.13^{a}	0.00 ± 0.00^{a}
	Protocorms	33.29 ± 0.38^{b}	28.94 ± 1.06^{a}	20.18 ± 0.39^{b}	11.65 ± 0.79^{b}	4.57 ± 0.23^{bc}	1.37 ± 0.23^{bc}
2,4-D	Protocorms	35.06 ± 2.39^{b}	29.37 ± 0.98^{a}	17.45 ± 1.32^{ab}	11.08 ± 0.98^{b}	5.09 ± 0.37^{b}	1.95 ± 0.40^{b}
TIBA	Protocorms	43.09 ± 0.46^{c}	29.36 ± 0.32^{a}	14.97 ± 0.10^{a}	8.49 ± 0.25^{a}	3.14 ± 0.17^{ad}	0.95 ± 0.08^{c}

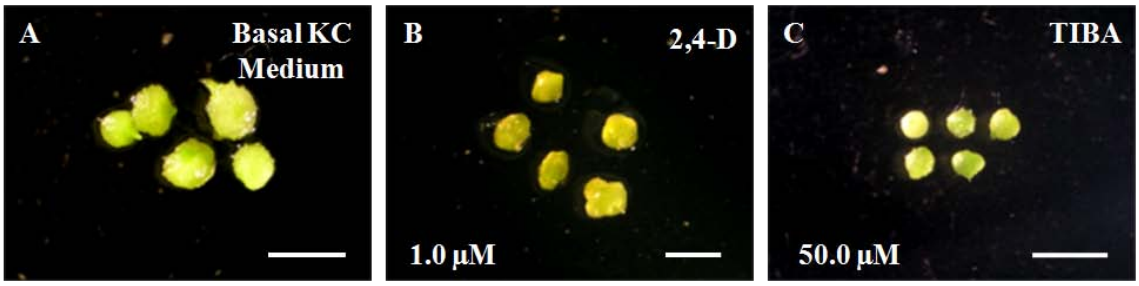

Fig 4.3. Protocorms of *D.* Chao Praya Smile cultured for 3 weeks in liquid basal KC medium or medium supplemented with 2,4-D or TIBA, bar: 0.3 cm.

A: Basal KC medium.

B: Basal KC medium with 2,4-D.

C: Basal KC medium with TIBA.

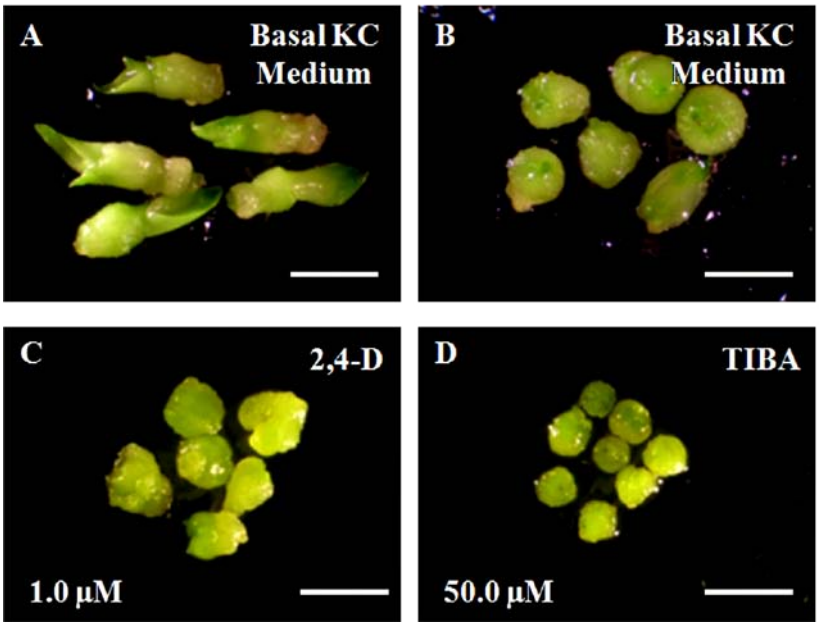

Fig 4.4. Protocorms of *D.* Chao Praya Smile cultured for 6 weeks in liquid basal KC medium or medium supplemented with 2,4-D or TIBA, bar: 0.5 cm.

A , B: Basal KC medium (**A**: Protocorms with leaves).

C: Basal KC medium with 2,4-D.

D: Basal KC medium with TIBA.

4.3.5 Effect of PAC

In this study, PAC was dissolved in absolute ethanol. Therefore, endopolyploidy profile of protocorms cultured in medium containing 2.0 ml l^{-1} absolute ethanol was analysed for comparison. Protocorms cultured in medium supplemented with 2.0 μM PAC were found to contain significantly less 2C and 4C nuclei (about 4 and 1 % less, respectively) and more 8C, 16C and 32C nuclei (about 2, 2 and 1 % more, respectively) as compared to the protocorms cultured in medium with 2.0 ml l^{-1} absolute ethanol for 3 weeks (Table 4.5; Fig 4.5). For protocorms cultured in medium containing 10.0 μM GA_3 for 3 weeks, they contained less 2C nuclei (about 5 % less) and more 8C nuclei (about 4 % more) than those cultured in basal KC medium (Table 4.5; Fig 4.5). Therefore, the increase in the mean frequency of 8C nuclei in protocorms cultured in medium containing 10.0 μM GA_3 was greater than those cultured in medium supplemented with 2.0 μM PAC for 3 weeks (Table 4.5).

After 6 weeks of culture, the mean frequency of 2C nuclei in the protocorms cultured in medium containing 2.0 μM PAC was significantly higher than those cultured in medium with 2.0 ml l^{-1} absolute ethanol (Table 4.5; Fig 4.6). On the other hand, no significant difference was observed in the mean frequencies of the nuclei with higher than 2C DNA content in the protocorms cultured in medium containing 2.0 μM PAC as compared to those cultured in medium with 2.0 ml l^{-1} absolute ethanol (Table 4.5).

In addition, no difference was observed in the distributions of the various nuclei in the protocorms cultured in medium containing 2.0 μM PAC for 3 and 6 weeks (Table 4.5).

Table 4.5. Effects of GA_3 and PAC on the pattern of endopolyploidy in the protocorms of *D.* Chao Praya Smile cultured in liquid basal KC medium and KC medium supplemented with 10.0 µM GA_3, 2.0 ml l^{-1} absolute ethanol or 2.0 µM PAC, based on flow cytometric analyses of 10 000 nuclei per replicate. Within the same week of culture, different letters following the standard error for the same C-values indicate significant difference based on Tukey's test at 95 % confidence interval.

Plant Growth Regulator / Solvent	Type of Culture	Mean Proportion of Nuclei (%) ± SE					
		2C	4C	8C	16C	32C	64C
3 Weeks After Culture							
-	Protocorms	43.30 ± 0.89^{a}	33.76 ± 0.25^{a}	13.85 ± 0.35^{a}	5.92 ± 0.21^{a}	2.32 ± 0.11^{a}	0.85 ± 0.15^{a}
GA_3	Protocorms	38.48 ± 0.66^{b}	36.69 ± 0.51^{b}	17.77 ± 0.42^{c}	5.75 ± 0.16^{a}	1.31 ± 0.13^{b}	0.00 ± 0.00^{b}
Ethanol	Protocorms	41.57 ± 0.45^{a}	32.21 ± 0.27^{b}	15.71 ± 0.13^{b}	7.04 ± 0.39^{b}	2.54 ± 0.12^{a}	0.93 ± 0.07^{a}
PAC	Protocorms	37.50 ± 0.48^{b}	30.87 ± 0.19^{c}	17.83 ± 0.29^{c}	8.80 ± 0.21^{c}	3.60 ± 0.13^{c}	1.22 ± 0.11^{a}
6 Weeks After Culture							
-	Protocorms with Leaves	41.88 ± 1.04^{a}	30.95 ± 0.37^{ac}	16.84 ± 1.47^{a}	7.71 ± 0.21^{a}	2.62 ± 0.13^{ac}	0.00 ± 0.00^{a}
	Protocorms	33.29 ± 0.38^{bh}	29.94 ± 1.06^{c}	20.18 ± 0.39^{abcd}	11.65 ± 0.79^{bd}	4.57 ± 0.23^{b}	1.37 ± 0.23^{bc}
GA_3	Elongated Protocorms	22.57 ± 0.76^{eg}	34.86 ± 0.38^{ab}	26.67 ± 0.21^{ef}	11.24 ± 0.94^{abd}	3.67 ± 0.33^{ab}	0.99 ± 0.18^{cd}
	Protocorms	26.00 ± 0.40^{de}	36.38 ± 1.31^{a}	24.17 ± 1.15^{df}	10.39 ± 0.14^{abd}	2.61 ± 0.21^{ac}	0.45 ± 0.10^{d}
Ethanol	Protocorms	28.65 ± 0.79^{cd}	31.59 ± 1.54^{abc}	21.09 ± 0.61^{bcd}	12.73 ± 1.03^{d}	4.83 ± 0.48^{b}	1.11 ± 0.10^{bc}
PAC	Protocorms	35.92 ± 0.67^{fh}	30.44 ± 0.54^{bc}	19.03 ± 0.41^{abc}	9.62 ± 0.26^{abd}	3.74 ± 0.07^{ab}	1.25 ± 0.19^{bc}

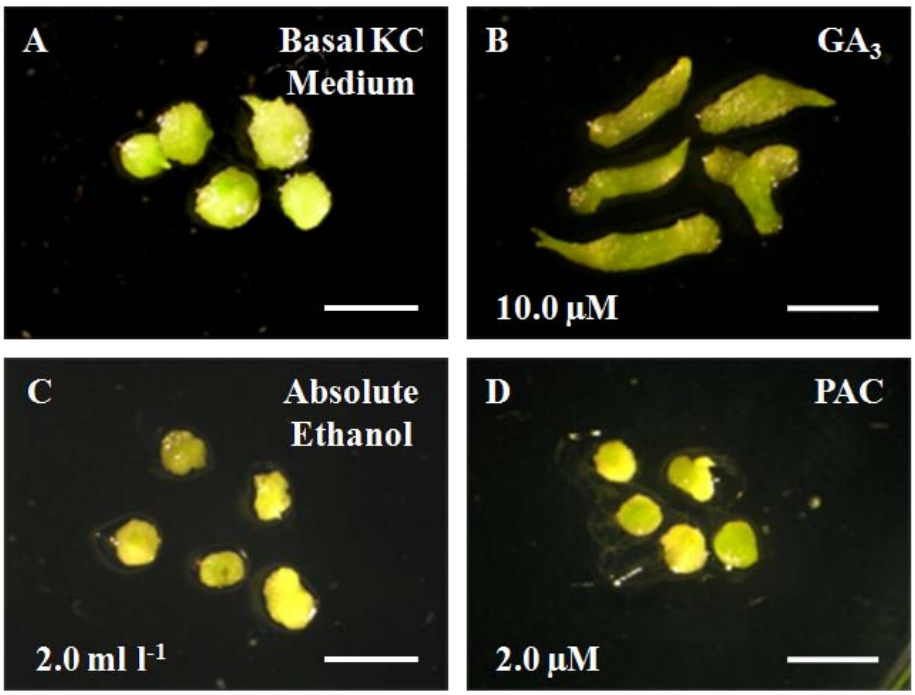

Fig 4.5. Protocorms of *D.* Chao Praya Smile cultured for 3 weeks in liquid basal KC medium or medium supplemented with GA_3, absolute ethanol or PAC, bar: 0.3 cm.

A: Basal KC medium.

B: Basal KC medium with GA_3.

C: Basal KC medium with absolute ethanol.

D: Basal KC medium with PAC.

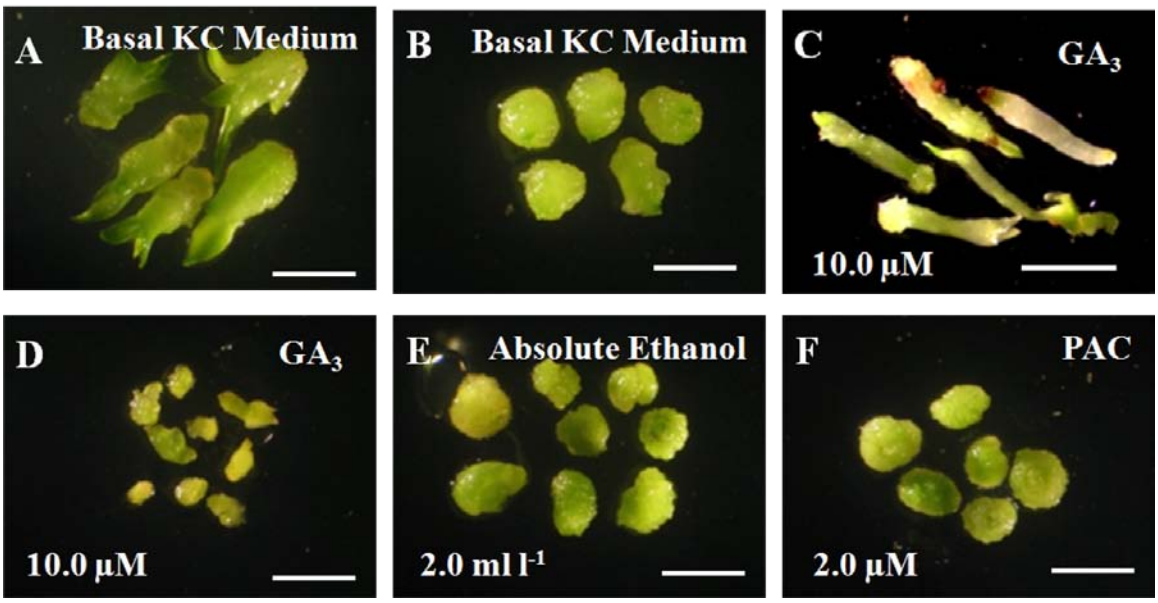

Fig 4.6. Protocorms of *D.* Chao Praya Smile cultured for 6 weeks in liquid basal KC medium or medium supplemented with GA_3, absolute ethanol or PAC, bar: 0.5 cm.

A , B: Basal KC medium (**A**: Protocorms with leaves).

C , D: Basal KC medium with GA_3 (**C**: Elongated protocorms).

E: Basal KC medium with absolute ethanol.

F: Basal KC medium with PAC.

4.4 Discussion

4.4.1 Effect of BA

Cytokinins are generally recognised as a requirement for the completion of mitosis and cytokinesis in plant cells (Jouanneau and De Marsac 1973, Liscum and Hangarter 1991). The presence of cytokinin in the culture medium often results in the reduction of endoreduplication in the cells. For instance, in the suspension cell cultures of the orchid *Doritaenopsis*, culture medium containing BA or thidiazuron reduced endoreduplication in the cells as indicated by the decrease in the mean frequency of 8C and 16C nuclei and an increase in 4C nuclei in the cells (Mishiba et al. 2001). In tobacco cells cultured in medium containing only auxin, cell elongation and an increase in endoreduplication were observed. However, medium containing both cytokinin and auxin induced cell division and reduced endoreduplication in the tobacco cells (Valente et al. 1998).

In this present study, a decrease in endopolyploidy was observed in the protocorms of *D.* Chao Praya Smile cultured in media containing BA for 3 weeks compared to the protocorms cultured in basal KC medium (Table 4.1). The inhibitory effect of BA on endopolyploidy in the protocorms of *D.* Chao Praya Smile cultured in media containing BA increased with increasing concentrations of BA in the media (Table 4.1). This implies that the effect of cytokinin on endopolyploidy in the protocorms is possibly dosage dependent. Moreover, higher ploidy variation was observed in the protocorms cultured in 10.0 µM BA for 6 weeks compared to that cultured in the same medium for 3 weeks (Table 4.1). This demonstrated that the presence of BA in the culture medium did not result in the total inhibition of endoreduplication in the

protocorms, suggesting that other factors may be involved in the regulation of this process.

It was reported that the levels of endogenous auxin and cytokinin would influence the activity of protein kinase, $p34^{cdc2}$, which might be involved in the regulation of endoreduplication (John et al. 1993, Nagl 1993, Valente et al. 1998). Endoreduplication was induced in pea root cortex cells cultured in medium containing both cytokinin and auxin. However, in medium containing auxin alone, no effect on endopolyploidy was observed (Libbenga and Torrey 1973). In maize endosperm, a sharp decline in the cytokinin/auxin ratio was found to coincide with an increase in endoreduplication (Lur and Setter 1993). This implies that endoreduplication may be affected by the balance of endogenous cytokinin-to-auxin ratio rather than the absolute concentration of cytokinin or auxin. The addition of cytokinin or auxin to the culture medium may have affected endoreduplication in the protocorms by altering the cytokinin/auxin ratio within the tissues. Further analyses on the changes in the endogenous auxin and cytokinin levels of the protocorms after culturing in medium containing cytokinin or auxin are needed to elucidate this.

4.4.2 Effect of 2,4-D

Auxin, such as 2,4-D, has been reported to increase chromosome instability of cells in cultures when incorporated in culture medium at high concentration (Gould 1986, Karp 1994). Auxin has also been reported to affect endopolyploidy in orchid cultures (Mishiba et al. 2001, Lim and Loh 2003). In this present study, culture media containing 2,4-D resulted in an increase in endopolyploidy in the protocorms of *D.* Chao Praya Smile as compared to those cultured in basal KC medium (Table 4.2). This is consistent with Lim and Loh (2003) who reported an increase in

endopolyploidy in the protocorms of *V.* Miss Joaquim cultured in medium containing 10.0 µM NAA. In the suspension cells of *Doritaenopsis,* the presence of 4.5 µM 2,4-D or 20.7 µM picloram in the culture medium resulted in an increase in endoreduplication in the cells (Mishiba et al. 2001).

In maize, the application of lanolin paste containing 2,4-D on the exposed pericarp surface of kernel at 5 or 7 days after pollination resulted in a rapid increase in endoreduplication in the endosperm (Lur and Setter 1993). This coincided with the accumulation of zein storage materials (indicator of cellular differentiation) in the maize endosperm (Lur and Setter 1993). These results demonstrated that the application of 2,4-D hastened the development of endosperm via the increase in endoreduplication (Lur and Setter 1993). Since the parenchymatous region of the orchid protocorms was suggested to be analogous to the endosperm of other angiosperms (Alvarez 1968), culturing of *D.* Chao Praya Smile protocorms in medium containing auxin might have hastened the protocorm development by increasing endoreduplication in the parenchymatous region. However, further investigation on the cellular differentiation of protocorms cultured in medium containing auxin is required to establish such a link.

4.4.3 Effect of GA_3

GAs control a range of growth and developmental processes in plants (Yamaguchi 2008). They have been reported to release seed dormancy and promote seed germination in tobacco (Leubner-Metzger 2001), enhance vegetative growth in maize (Evans and Poethig 1995) and promote flowering in *B. napus* (Rood et al. 1989) and *Arabidopsis* (Tyler et al. 2004), just to name a few. At the cellular level, GAs have

been reported to promote cell division and elongation (Kende and Zeevaart 1997) and are involved in endoreduplication (Joubes and Chevalier 2000).

In the present study, GA_3 resulted in a slight increase in endoreduplication in the protocorms of *D.* Chao Praya Smile cultured in medium containing 10.0 µM GA_3 for 3 weeks (Table 4.3). This is consistent with Lim and Loh (2003) who reported a slight increase in endopolyploidy in the protocorms of *V.* Miss Joaquim cultured in medium containing 10.0 µM GA_3. In GA-deficient mutants of *Arabidopsis*, the hypocotyls exhibited reduced ploidy level, while an addition of GA to the culture medium restored the ploidy level to that of the wild-type (Gendreau et al. 1999). On the other hand, the enhancing effect of GA on endopolyploidy was found to be cultivar-dependent in *Pisum sativum* (Callebaut et al. 1982) and *Triticum durum* (Cavallini et al. 1995).

The effect of GA on endoreduplication seems to be dependent on other environmental factors as well. For instance, the presence of GA_3 resulted in an increase in endopolyploidy in the leaf cells of *T. durum* cultivated in the dark (Cavallini et al. 1995). However, no increase in endopolyploidy in the leaf cells was observed when the plants were grown in the light (Cavallini et al. 1995). This implies that the effect of GAs on endopolyploidy could be the consequence of the responses to GAs rather than a direct effect on endoreduplication (Gendreau et al. 1999).

In the protocorms of *D.* Chao Praya Smile, a further increase in endopolyploidy was resulted when cultured in medium containing 10.0 µM GA_3 for 6 weeks (Table 4.3). At the same time, protocorms with leaves were not observed and elongation of protocorms was apparent after culturing in medium containing 10.0 µM GA_3 for 6 weeks (Fig 4.2O). Endopolyploidy in these elongated protocorms was found to be

higher than non-elongated protocorms in the same medium (Table 4.3). This suggests that the elongation of protocorms could be due to the increase in endopolyploidy which in turn increased the cell size of the protocorms. Several studies had found positive correlation between cell size and variation in endopolyploidy (Melaragno et al. 1993, Folkers et al. 1997, Cebolla et al. 1999, Kondorosi et al. 2000). Endoreduplication was also suggested to be required for the expansion and differentiation of plant cells (Kondorosi et al. 2000, Barow 2006). On the other hand, cell elongation was reported to be uncoupled from endoreduplication in *Arabidopsis* (Gendreau et al. 1998). The root cells from different ecotypes of *Arabidopsis* had varied sizes, but no correlation was found between cell size and ploidy level (Beemster et al. 2002). Therefore, determining the nuclear and cell size of the protocorms of *D.* Chao Praya Smile cultured in media containing varying concentrations of GA_3 would be useful in verifying the correlation between cell size and endopolyploidy variation in the protocorms.

4.4.4 Effect of TIBA

During tissue culture, an increase in ploidy variations in the cultures was reported to result in the loss of regenerative ability of cells (Gould 1986). For instance, in *Rosa hydrida* tissue cultures, an increase in the number of polyploid cells would result in a decline in regeneration potential (Moyne et al. 1993). The addition of TIBA to the culture medium, however, would enhance shoot regeneration from the low-regenerative callus cultures of *R. hydrida* (Singh and Syamal 2000). This implies that TIBA may have reduced the ploidy level in the cells to restore their regeneration potential.

TIBA is an auxin polar transport inhibitor and has been used to study the role of auxin in plant development (Dhonukshe et al. 2008). Auxin polar transport inhibitors (Katekar and Geissler 1980) and inhibitor of auxin action (α-(p-chlorophenoxy) isobutryic acid (PCIB)) (Oono et al. 2003) have antagonistic effects on auxin activity. Some of the reported effects of these inhibitors include inhibition of root growth (Ramanayake et al. 2008) and lost of apical dominance in plants (Ishikawa et al. 1997). In maize endosperm, application of lanolin paste supplemented with PCIB to the exposed pericarp surface of the kernel resulted in a significant decrease in endopolyploidy in the endosperm (Lur and Setter 1993). However, the effect of TIBA on endopolyploidy in plants has yet to be reported.

In this study, *D.* Chao Praya Smile protocorms cultured in medium containing 50.0 µM TIBA for 3 weeks did not result in a significant decrease in endopolyploidy compared to those cultured in basal KC medium (Table 4.4). However, the ploidy variation in the protocorms cultured in medium containing 50.0 µM TIBA for 6 weeks was significantly lower than those cultured in basal KC medium (Table 4.4). Moreover, endopolyploidy variations in the protocorms cultured in medium containing 50.0 µM TIBA for 3 and 6 weeks were similar (Table 4.4). This suggests that TIBA inhibited further increase in endoreduplication in the protocorms after 3 weeks of culture. However, direct effect on endoreduplication in the cells via TIBA has yet to be reported. TIBA is recognised to have an antagonistic effect on auxin activity (Katekar and Geissler 1980). Therefore, it is possible that the presence of TIBA in the culture medium might have inhibited auxin activity in the protocorms which in turn inhibited endoreduplication. Further study into the regulatory mechanism of TIBA on endoreduplication will therefore aid in the understanding of the role of TIBA in this process.

4.4.5 Effect of PAC

The study on the effect of endogenous GAs levels on endoreduplication in the protocorms is necessary to further understand the role of GA on endoreduplication. Endogenous GAs levels can be reduced by four groups of GA biosynthesis inhibitors. These four groups are a) compounds with a nitrogen-containing heterocycle (triazole-type compounds), b) "onium" compounds, c) structural mimics of 2-oxoglutaric acid and d) 16,17-dihydo-GAs (Rademacher 2000). Each of these inhibitor groups inhibits a distinct step in the GA biosynthesis pathway (Rademacher 2000).

In this study, PAC, a triazole-type GA biosynthesis inhibitor, was used. As aforementioned, the presence of GA_3 in the culture medium was found to enhance endoreduplication in the protocorms of *D.* Chao Praya Smile (Table 4.3). The reduction of endogenous GAs levels using GA biosynthesis inhibitor would, therefore, inhibit this process. However, an increase in endopolyploidy was resulted in the protocorms cultured in medium containing 2.0 µM PAC for 3 weeks compared to the protocorms cultured in medium with 2.0 ml l^{-1} absolute ethanol (Table 4.5). On the other hand, this increase in endopolyploidy in the protocorms cultured in medium containing 2.0 µM PAC was lower than that in the protocorms cultured in medium supplemented with 10.0 µM GA_3 for 3 weeks (Table 4.5).

Desgagne-Penix and Sponsel (2008) reported that the presence of 5.0 µM PAC in culture medium would increase the expression of GA20-oxidase mRNAs (*AtGA20ox1*) in the cotyledons and leaves of 6-day-old *Arabidopsi*s seedlings. GA20-oxidase catalyses the conversion of inactive GA (GA_{20}) to bioactive GA (GA_1) (Fig 4.7) and over-expression of *AtGA20ox1* in the tissues of transgenic *Arabidopsi*s plants was found to increase the endogenous levels of bioactive GAs (Coles et al. 1998, Huang et

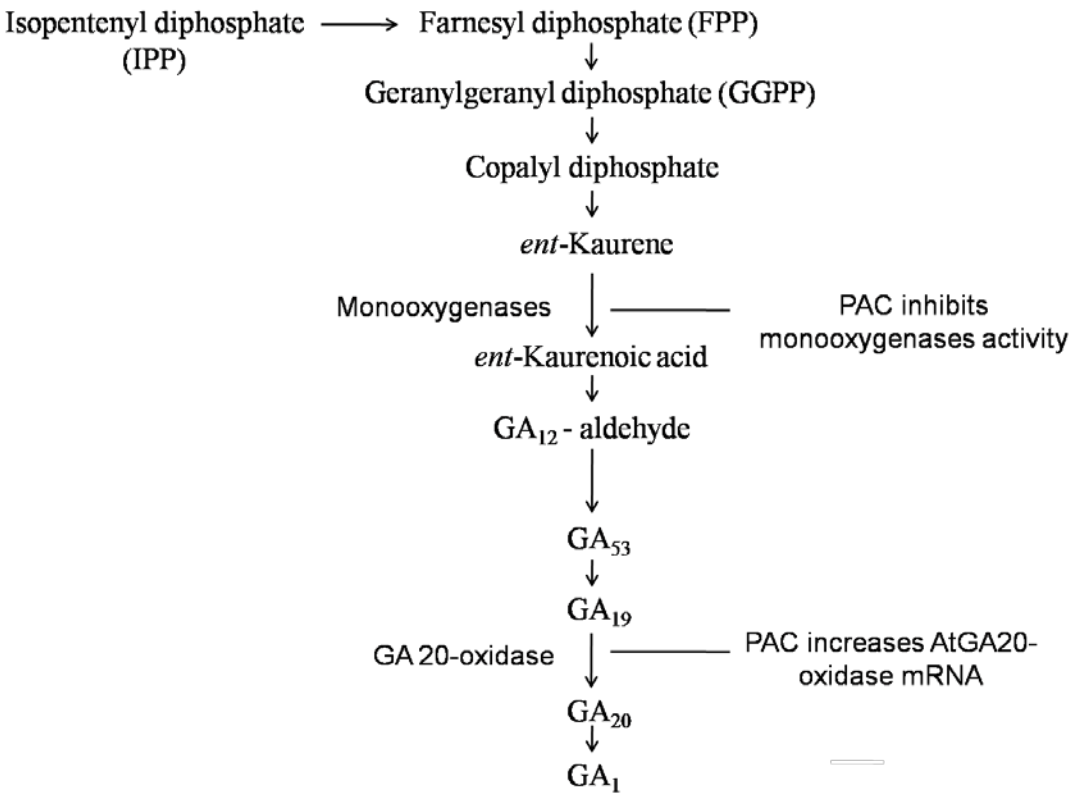

Fig 4.7. A simplified GA biosynthesis pathway showing the points of PAC activities (adapted from Rademacher 2000).

al. 1998). Therefore, in this present study, the presence of 2.0 µM PAC in the culture medium could have induced the expression of GA20-oxidase mRNAs in the protocorms of *D.* Chao Praya Smile. This probably increased the conversion of the remaining endogenous inactive GAs into their active forms (Fig 4.7) and resulted in the initial increase in endopolyploidy observed in the protocorms cultured in medium containing 2.0 µM PAC for 3 weeks (Table 4.5).

In contrast, no further increase in endopolyploidy was observed in the protocorms of *D.* Chao Praya Smile cultured in medium containing 2.0 µM PAC for 6 weeks as compared to those cultured in the same medium for 3 weeks (Table 4.5). PAC reduces the production of endogenous GA by inhibiting the activity of monooxygenases which catalyse the oxidative steps from *ent*-Kaurene to *ent*-Kaurenoic acid (Fig 4.7) (Graebe 1987, Kende and Zeevaart 1997, Rademacher 2000). Further synthesis of the inactive GAs in the protocorms cultured in medium containing 2.0 µM PAC would be prevented. This could result in the decrease in the levels of bioactive GAs in these protocorms after the initial increase. The decrease in the levels of bioactive GAs would possibly inhibit endoreduplication in these protocorms.

In conclusion, the presence of BA in the culture medium was found to reduce endoreduplication, while 2,4-D and GA enhanced this process in the protocorms of *D.* Chao Praya Smile. Unlike BA, the addition of TIBA or PAC to the culture medium inhibited endoreduplication and further increase in endopolyploidy (due to development) was not observed in the protocorms cultured in these media for 6 weeks. These results are consistent with the suggestion that plant growth regulators would affect the genetic stability of the tissues by influencing the cell cycle or endoreduplication cycle (Brar and Jain 1998). However, the effects of some of these

growth regulators in endoreduplication in the plant tissues varied in different plants (Callebaut et al. 1982, Cavallini et al. 1995, Joubes and Chevalier 2000). It has been suggested to be cultivar dependent in plants such as *P. sativum* (Callebaut et al. 1982) and *T. durum* (Cavallini et al. 1995). It could also be due to the nuclear stability in the plant cells before culturing in medium containing plant growth regulator. To provide more evidence on this, further investigations into the effects of plant growth regulators on endoreduplication in the tissues from plants which exhibited minimum ploidy variations are required.

Chapter 5 Tissue culture and endopolyploidy in *A. andraeanum* cv 'Red Hot'

5.1 Introduction

Beside orchids, anthuriums are the other horticulturally important plants. In the global market, anthurium is second only to orchid among the tropical cut flowers (Dufour and Guerin 2003). *A. andraeanum* is a highly cultivated *Anthurium* species because of its large and showy spathes (Henny 1999). Due to their popularity, specific small hybrids such as *A. andraeanum* cv 'Lady Jane', 'Southern Blush' and 'Red Hot' have been developed as potted plants (Henny et al. 1988, Henny 1999).

Some *Anthurium* cultivars fail to produce viable seeds due to incompatibilities **(**Sheffer and Kamemoto 1976a). For those that produce viable seeds, propagation via seeds led to progenies that are heterozygous (Matsumoto et al. 1998, Martin et al. 2003). Therefore, anthuriums are usually propagated through asexual means (Matsumoto et al. 1998, Martin et al. 2003). However, conventional propagation method via separation from adult anthurium plants is time consuming and inefficient in developing commercial quantities of the elite clone (Pierik et al. 1974). To solve these problems, micro-propagation has been widely used.

Due to the difficulty in obtaining large numbers of nodal explants, anthuriums are normally micro-propagated using explants such as leaf, petiole, spathe and spadix via indirect organogenesis (Martin et al. 2003). Micro-propagation of plants via organogenesis through callus culture may risk the presence of somaclonal variants (Larkin and Scowcroft 1981). Such variations could be due to karyotype changes (endopolyploidy) which occur as a result of chromosomal replication in the absence of

cell and nuclear division (endoreduplication) during the normal process of cell division (Larkin and Scowcroft 1981). The extent of polyploidy in the cultures was suggested to be affected by the age of explant source or the use of plant growth regulators during tissue culture (D'Amato 1977, Sulistyaningsih et al. 2006).

The extent of endopolyploidy in the tissues also affects the regeneration potential of callus tissues (Gould 1986). For instance, increased occurrence of multiploid cells in cell suspension cultures of rose plants could decrease regeneration potential of the cells (Moyne et al. 1993). In asparagus, cells of callus cultures with high regeneration potential contained mostly 2C nuclei, while in non-organogenic callus cultures, 4C and 8C nuclei were detected (Reuther and Becker 1987).

Therefore, analyses on the endopolyploidy profiles of the tissues used in micro-propagation and the effects of plant growth regulators such as BA and 2,4-D on endopolyploidy in the callus tissues are required to provide an insight on the nuclear stability of the tissues of *A. andraeanum* plants and their callus cultures. Hence, the objectives of this chapter are: (1) to determine the optimum medium for callus induction, shoot regeneration and shoot multiplication; (2) to analyse the occurrence of multiploid cells within different tissues in greenhouse-grown plants and tissue-cultured *A. andraeanum* cv 'Red Hot' plantlets and (3) to study the effects of BA and 2,4-D on endopolyploidy in the callus tissues of *A. andraeanum* cv 'Red Hot'.

5.2 Materials and methods

5.2.1 Plant materials

Vegetatively propagated *A. andraeanum* cv 'Red Hot' (*A. andraeanum* cv 'Southern Blush' × *A. andraeanum* cv 'Lady Jane') were grown in pots in the greenhouse in the Department of Biological Sciences, National University of Singapore, at 28 ± 4 ºC with natural lighting of intensity 90.4 ± 12.4 µE m^{-2} s^{-1}.

Tissue-cultured plantlets

Young leaf laminas of *A. andraeanum* cv 'Red Hot' at the olive-brown unopened stage (see Fig 5.1A) were harvested and washed with detergent, surface sterilised by soaking in 80 % (v/v) ethanol for 2 min, followed by 20 % (v/v) Clorox^{TM} solution with a drop of Tween 20 for 15 min, with constant agitation. The lamina segments were then rinsed five times with autoclaved water. The sterilised leaf laminas were cut into 1-cm^2 and cultured in 90 mm petri dishes containing 25 ml of basal medium (modified Murashige and Skoog (1962) media (MS) containing: half-strength MS macro-elements and full strength MS micro-elements, iron and vitamins) supplemented with 4.44 µM BA and varying concentrations of 2,4-D, indole-3-acetic acid (IAA) or NAA, 2 % (w/v) sucrose and solidified with 0.3 % (w/v) gelrite at pH 5.5.

Greenhouse-grown plants and tissue-cultured plantlets for endopolyploidy analyses

For flow cytometric analyses of greenhouse-grown plants, petioles and laminas (proximal and distal lamina sections) of young unopened leaves with 5-cm long petioles and fully expanded leaves (30 days after opening) (see Fig 5.1A) were analysed. For roots, entire of 2- and 8-cm long roots were chosen for sample

preparation. For flowers, spadices and spathes of flowers at 10 days before anthesis (with 10-cm long peduncles) and at 30 days after anthesis (see Fig 5.1B) were analysed. For tissue-cultured plantlets, petioles and laminas (proximal and distal lamina sections) of young unopened leaves (with 2-cm long petioles) as well as fully expanded leaves (30 days after opening) and entire of 8-cm long roots were analysed.

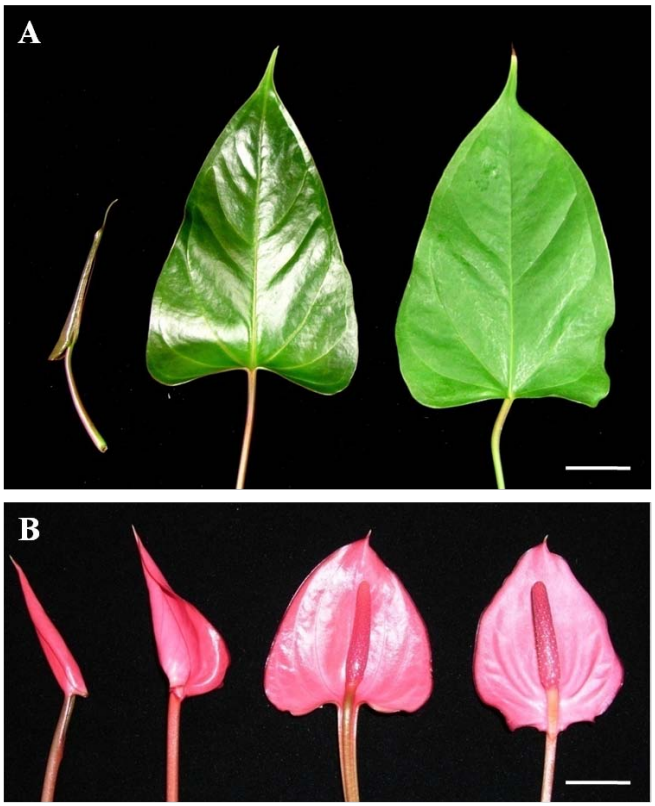

Fig 5.1. Developmental stages of greenhouse-grown *A. andraeanum* cv 'Red Hot' leaves and flowers.

A: Leaves at different developmental stages, bar: 2.24 cm.
Left to right: Unopened, newly opened and fully expanded leaves (fully expanded for 30 days).

B: Flowers at different days of anthesis, bar: 1.92 cm.
Left to right: Ten days before anthesis (with 8- to 10-cm peduncle), 1 day before anthesis, 3 days and 30 days after anthesis.

5.2.2 Callus induction and shoot regeneration

Young unopened lamina segments (1-cm^2) and petioles (1-cm long) from tissue-cultured plantlets were used as explants for callus induction. The explants were cultured in 90 mm petri dishes containing 25 ml of basal media supplemented with 4.44 µM BA and 2,4-D at four concentrations (0, 1.13, 2.26, 4.52 µM), 2 % (w/v) sucrose and solidified with 0.3 % (w/v) gelrite at pH 5.5.

After 3 months of culture, explants with callus were used for shoot regeneration. Callus explants were randomly transferred into 90 mm petri dishes containing 25 ml of basal media supplemented with 4.44 µM BA and 2,4-D at four concentrations (0, 1.13, 2.26, 4.52 µM), 2 % (w/v) sucrose and solidified with 0.3 % (w/v) gelrite at pH 5.5.

5.2.3 Shoot multiplication

Tissue-cultured plantlets about 5-cm tall (measured from youngest leaf tip to the base of the plant) with three fully expanded leaves were used. Roots from the plantlets were trimmed and transferred into Magenta GA7^{TM} containers containing 50 ml of basal media supplemented with BA of varying concentrations, 2 % (w/v) sucrose and solidified with 0.3 % (w/v) gelrite at pH 5.5.

Tissue-cultured plantlets were also cultured in Magenta GA7^{TM} containers containing 50 ml of basal media supplemented with 11.1 µM of cytokinin (BA, zeatin, 6-(γ-γ-dimethylallylamino) purine (2-iP) or kinetin), 2 % (w/v) sucrose and solidified with 0.3 % gelrite at pH 5.5. Unless otherwise stated, all cultures were maintained at 25 ºC under 16 hours illumination of intensity 45.9 ± 1.3 µE m^{-2} s^{-1}. Subculturing was done every 3 weeks.

5.2.4 Measurement of plant growth and development

The lengths of leaves and petioles of greenhouse-grown plants and tissue-cultured plantlets as well as spathes and peduncles of greenhouse-grown plants were measured for a period of 40 to 50 days. Measurements were first made (Day 0 of growth) when the petioles or peduncles were about 0.5- to 1.0-cm long. Time taken for the leaves and spathes to be fully expanded was recorded.

5.2.5 Effects of plant growth regulators on endopolyploidy in the callus tissues

Callus cultures were maintained in liquid basal medium (see section 5.2.1) for 6 weeks. These callus tissues were then transferred into liquid basal media supplemented with BA, or 2,4-D of varying concentrations. The cultures were maintained on a rotary shaker at 120 rpm at 25 ºC under 16 hours illumination of intensity 45.9 ± 1.3 µE m^{-2} s^{-1}. Subculturing was done every 3 weeks. Callus tissues were harvested after culturing in liquid media containing BA or 2,4-D for 6 weeks and flow cytometric analyses were conducted.

5.2.6 Nuclei isolation for flow cytometric analyses

Nuclei were extracted according to section 3.2.2 with some modifications. Tissue samples were chopped with a sterile doubled-sided razor blade in extraction buffer; 0.03 g tissue to 1 ml extraction buffer ratio for spadix, 0.15 g tissue to 1 ml extraction buffer ratio for callus tissues and 0.08 g of tissue to 1 ml of buffer ratio for other sample types.

5.2.7 Flow cytometric analyses

Method described in section 3.2.3 was used for flow cytometric analyses.

5.2.8 Statistical analyses

All percentage data were analysed with one-way analysis of variance (ANOVA) and Tukey's test at 95 % confidence level was performed for multiple pair-wise comparisons. Data were arcsine-transformed to meet assumptions of ANOVA (normality and homogeneity of variance) and analysed with Minitab™ 15.

5.3 Results

5.3.1 Effects of auxins on callus induction and shoot regeneration

Callus induction from young leaf lamina explants

All leaf lamina explants from greenhouse-grown plants expanded and became double in size within 2 weeks of culturing on media supplemented with 4.44 µM BA and varying concentrations of 2,4-D, IAA or NAA. Their colour changed from olive-brown to dark green. After culturing in these media for 2 months, calli began to develop at the cut end of some explants (Fig 5.2A). 33.33 % of the leaf lamina explants cultured on auxin-free medium produced callus. 64.86 % and 75 % of the leaf lamina explants cultured on media containing 2.26 µM 2,4-D and 5.71 µM IAA, respectively were observed to develop callus (Table 5.1). However, no callus was observed in the explants cultured on media supplemented with NAA (Table 5.1). After 3 months of culture, shoot buds were observed from the callus explants cultured on medium containing 2.26 µM 2,4-D (Fig 5.2B). Regeneration of shoots were observed in the leaf lamina explants after 6 months of culturing in medium supplemented with 4.44 µM BA and 2.26 µM 2,4-D (Fig 5.2C).

Table 5.1. Effects of auxins (2,4-D, IAA and NAA) on callus induction of young leaf lamina explants from greenhouse-grown *A. andraeanum* cv 'Red Hot' plants (n = 6 to 25 per replicate). (Duplicates were conducted and results were presented as the means of duplicates).

Concentration of Auxin (µM)			Percentage of Explants with Callus (%) ± SE
2,4-D	IAA	NAA	
-	-	-	33.33 ± 16.7
2.26	-	-	64.86 ± 20.9
4.52	-	-	43.75 ± 6.30
9.05	-	-	2.27 ± 2.28
-	2.85	-	45.83 ± 12.5
-	5.71	-	75.00 ± 25.0
-	11.4	-	25.00 ± 8.33
-	-	2.69	0.00 ± 0.00
-	-	5.37	0.00 ± 0.00
-	-	10.7	0.00 ± 0.00

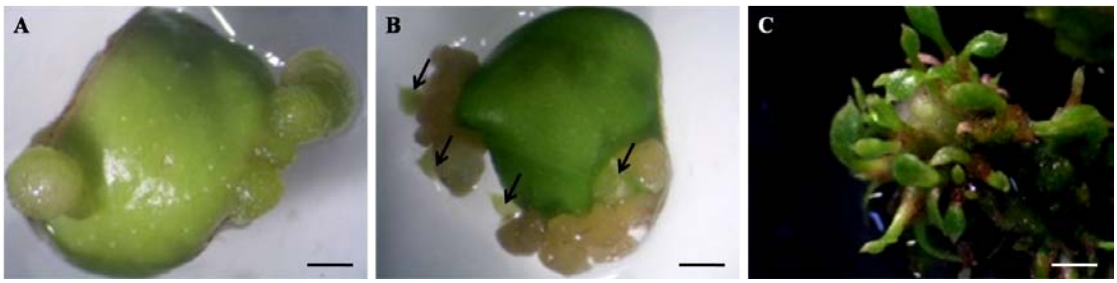

Fig 5.2. Young leaf lamina explants from greenhouse-grown *A. andraeanum* cv 'Red Hot' cultured on basal medium supplemented with 4.44 µM BA and 2.26 µM 2,4-D.

A: Two-month-old explants with callus, bar: 0.35 cm.

B: Three-month-old explants with shoot buds, arrows indicate shoot buds, bar: 0.4 cm.

C: Callus explants with regenerated shoots after 6 months of culture, bar: 0.12 cm.

Callus induction with young leaf laminas and petioles of tissue-cultured plantlets

Regenerants from the leaf laminas of greenhouse-grown plants were used for further experiments on callus induction and shoot regeneration. The unopened leaf laminas (proximal and distal sections) and petioles of the regenerants were used.

The lamina explants doubled in size after 2 weeks of culturing in media supplemented with 4.44 µM BA and varying concentrations of 2,4-D. After about 2 months, 86 % of petiole explants cultured on medium containing 1.13 µM 2,4-D produced callus (Fig 5.3A). In medium containing 4.52 µM 2,4-D, only 45 % of petiole explants was observed to produce callus (Fig 5.3A). In the proximal leaf lamina cultures, increasing concentrations of 2,4-D (0 to 4.52 µM) did not result in significant changes to the percentage of explants with callus. The percentage of explants with callus ranged from 23 to 63 % (Fig 5.3B). In the distal leaf lamina cultures, no callus was observed in the explants cultured on 2,4-D-free medium, while callus was observed in 44 % of the explants cultured on medium containing 2.26 µM 2,4-D (Fig 5.3C).

Shoot regeneration

Regeneration of shoots from the callus explants was observed about 3 months after subculturing to medium supplemented with 4.44 µM BA and varying concentrations of 2,4-D (Fig 5.4). The average number of shoots regenerated from callus tissues cultured on medium supplemented with 1.13 and 2.26 µM 2,4-D (21 and 16 shoots, respectively) were significantly higher, as compared to those cultured on 2,4-D-free medium and medium supplemented with 4.52 µM 2,4-D (Fig 5.4).

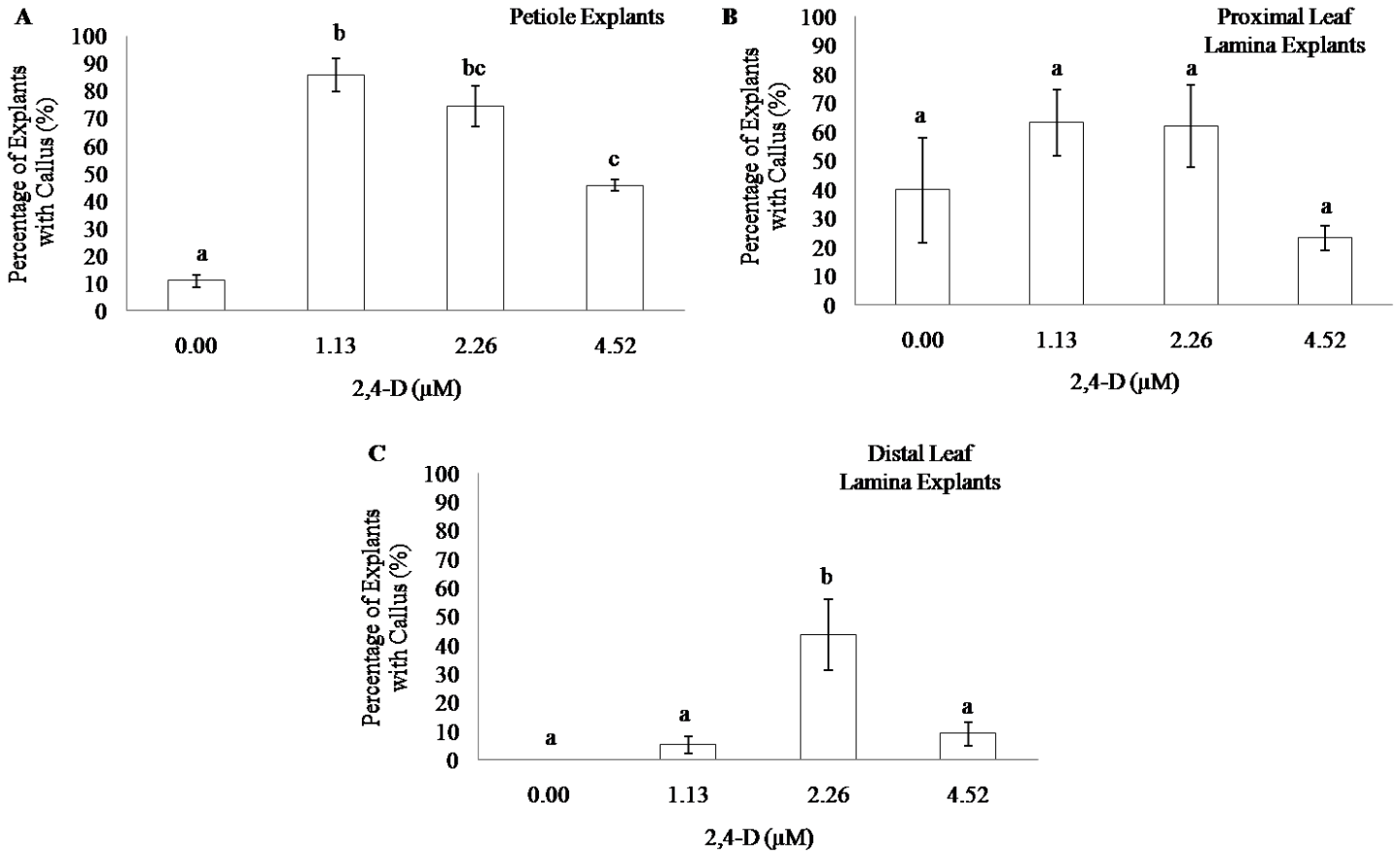

Fig 5.3. Effect of 2,4-D on callus induction of different types of explant from tissue-cultured *A. andraeanum* cv 'Red Hot' plantlets (n = 5 to 18 per replicate). A: Petiole explants. B: Proximal leaf lamina explants. C: Distal leaf lamina explants. Data were expressed as the mean of triplicates ± SE. Different letters on each bar indicate significant difference based on Tukey's test at 95 % confidence interval.

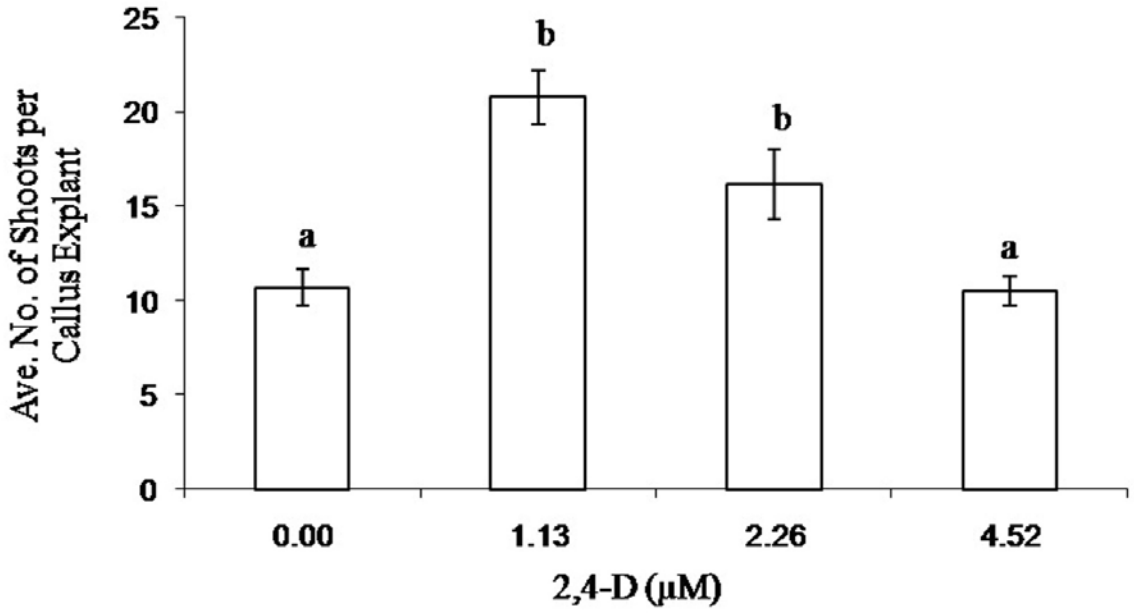

Fig 5.4. Effect of 2,4-D on shoot regeneration from callus explants cultured in media containing 4.44 µM BA and 2,4-D for 3 months. Data were expressed as the average of 8 to 12 replicates ± SE. Different letters on each bar indicate significant difference based on Tukey's test at 95 % confidence interval.

5.3.2 Effects of cytokinins on shoot multiplication of tissue-cultured plantlets

Plantlets cultured in medium containing 11.1 µM BA produced an average of 3 shoots after 28 days of culture and was significantly higher than those cultured with media containing other concentrations of BA (Fig 5.5A). About 6 leaves were produced in plantlets cultured in media containing 4.44 to 22.2 µM BA (Fig 5.5B). Roots were observed in all the plantlets grown in basal medium and an average of 3 roots per plantlet was produced (Figs 5.5C and 5.6A). In plantlets cultured in medium supplemented with 44.4 µM BA, roots were not observed (Figs 5.5C and 5.6E).

Plantlets cultured in medium containing 11.1 µM zeatin produced an average of 9 shoots per plantlet and was significantly higher as compared to those cultured with 11.1 µM BA, kinetin or 2-iP (Fig 5.7A). The average number of leaves produced per plantlet cultured in medium with 11.1 µM zeatin was 21 (Fig 5.7B).

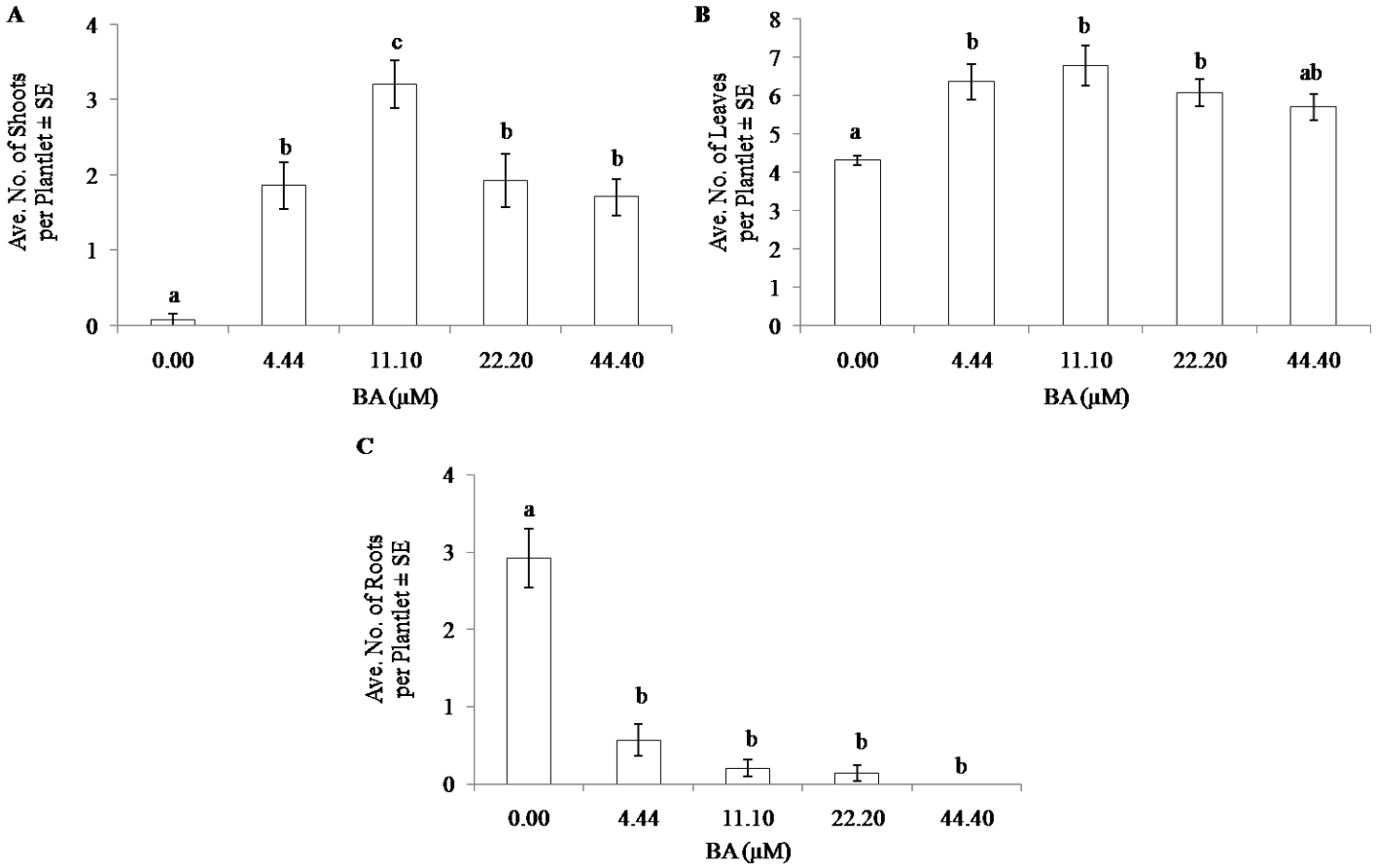

Fig 5.5. Effect of BA on shoot multiplication from tissue-cultured *A. andraeanum* cv 'Red Hot' plantlets cultured in media containing BA for 28 days. A: Average shoots per plantlet. B: Average leaves per plantlet. C: Average roots per plantlet (n = 15). Different letters on each bar indicate significant difference based on Tukey's test at 95 % confidence interval.

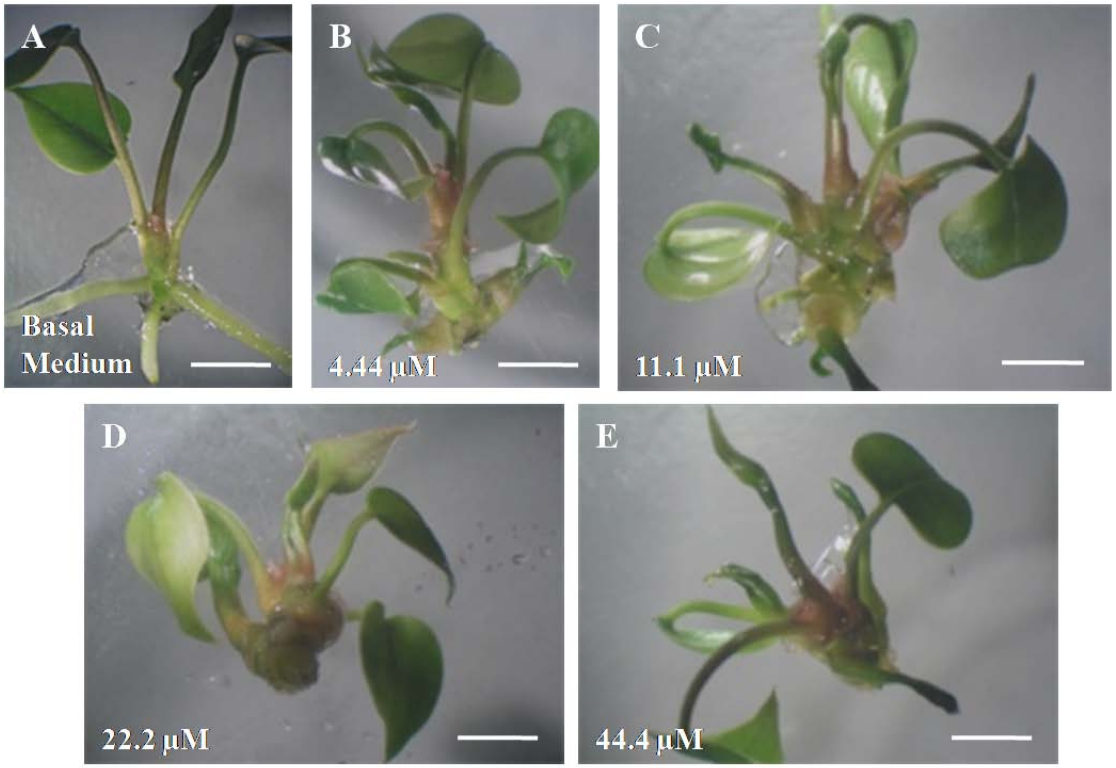

Fig 5.6. Shoots cultured for 28 days in basal medium or media supplemented with BA.

A: Basal medium, bar: 1.3 cm.

B: Basal medium with 4.44 µM BA, bar: 1.4 cm.

C: Basal medium with 11.1 µM BA, bar: 1.0 cm.

D: Basal medium with 22.2 µM BA, bar: 1.0 cm.

E: Basal medium with 44.4 µM BA, bar: 1.0 cm.

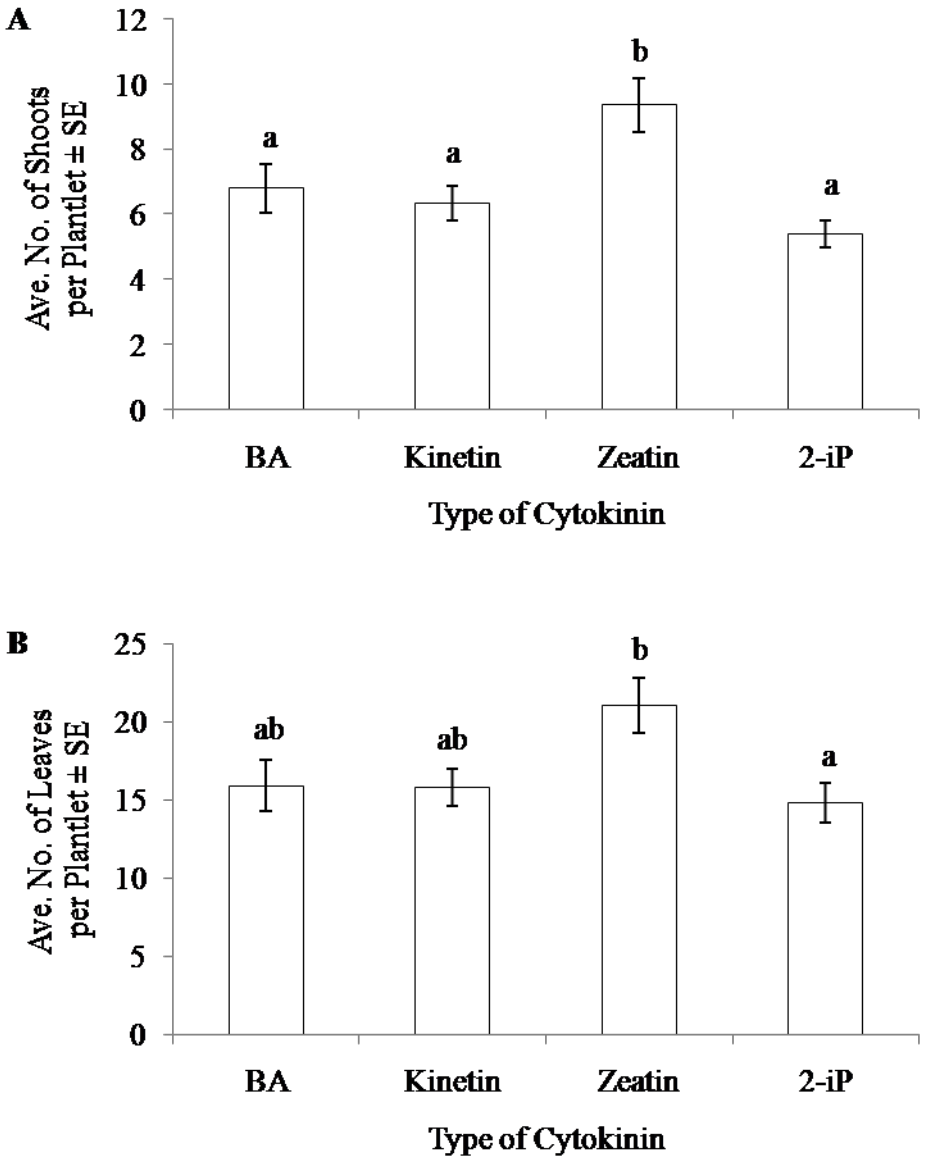

Fig 5.7. Effect of 11.1 µM of cytokinin (BA, kinetin, zeatin or 2-iP) on shoot multiplication and leaf production of tissue-cultured *A. andraeanum* cv 'Red Hot' plantlets cultured in media containing cytokinin for 56 days (n = 20). Different letters on each bar indicate significant difference based on Tukey's test at 95 % confidence interval.

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5.3.3 Growth patterns of greenhouse-grown plants and tissue-cultured plantlets

The growth patterns of petioles, laminas and peduncles of greenhouse-grown *A. andraeanum* cv 'Red Hot' plants followed a sigmoidal growth curve (Figs 5.8A - C) which consisted of an initial lag phase, followed by an exponential growth phase and lastly a stationary growth phase. The spathes of the plants showed a gradual increase in length (from an average of 1.7 to 4.7 cm) and reached stationary phase after 30 days of growth (Fig 5.8D).

Young leaves opened at around day 14 of growth and average length of their petioles was 6.7 cm (Fig 5.8). The colour of leaf laminas changed from olive-brown to green and eventually dark green when the leaf laminas were fully expanded (Fig 5.1A). The spathes of newly emerged flowers were unopened. The opening of spathes (anthesis) were observed at about 29 days of growth (Fig 5.8D).

The development of petioles and laminas of tissue-cultured plantlets also followed a sigmoidal growth curve (Fig 5.9). However, the lag and exponential growth phases were less distinct. The lag phase of leaf petioles was from day 0 to day 2 of growth. The lag phase of leaf laminas was not distinct. Both leaf petioles and laminas reached stationary growth phase on day 25 of growth. The leaves remained dark green from day 0 to day 40 of growth. The average length of the petioles when leaf laminas opened was 2.6 cm (Fig 5.9).

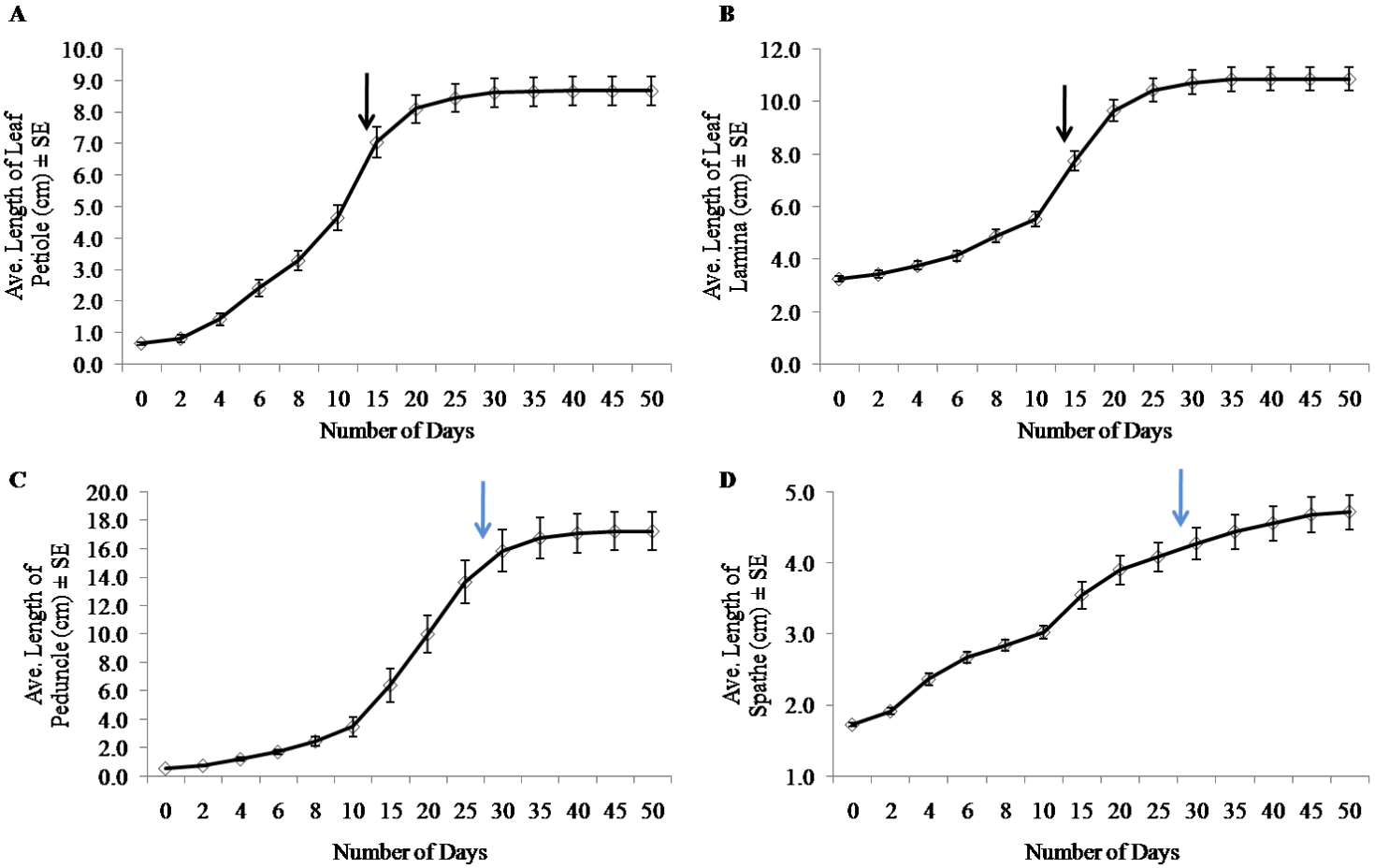

Fig 5.8. Growth curves of leaf petioles, laminas, peduncles and spathes of greenhouse-grown *A. andraeanum* cv 'Red Hot' plants (n = 25). A: Leaf petiole. B: Leaf lamina. C: Peduncle. D: Spathe. Black arrows: Leaf opened; Blue arrows: Day of anthesis.

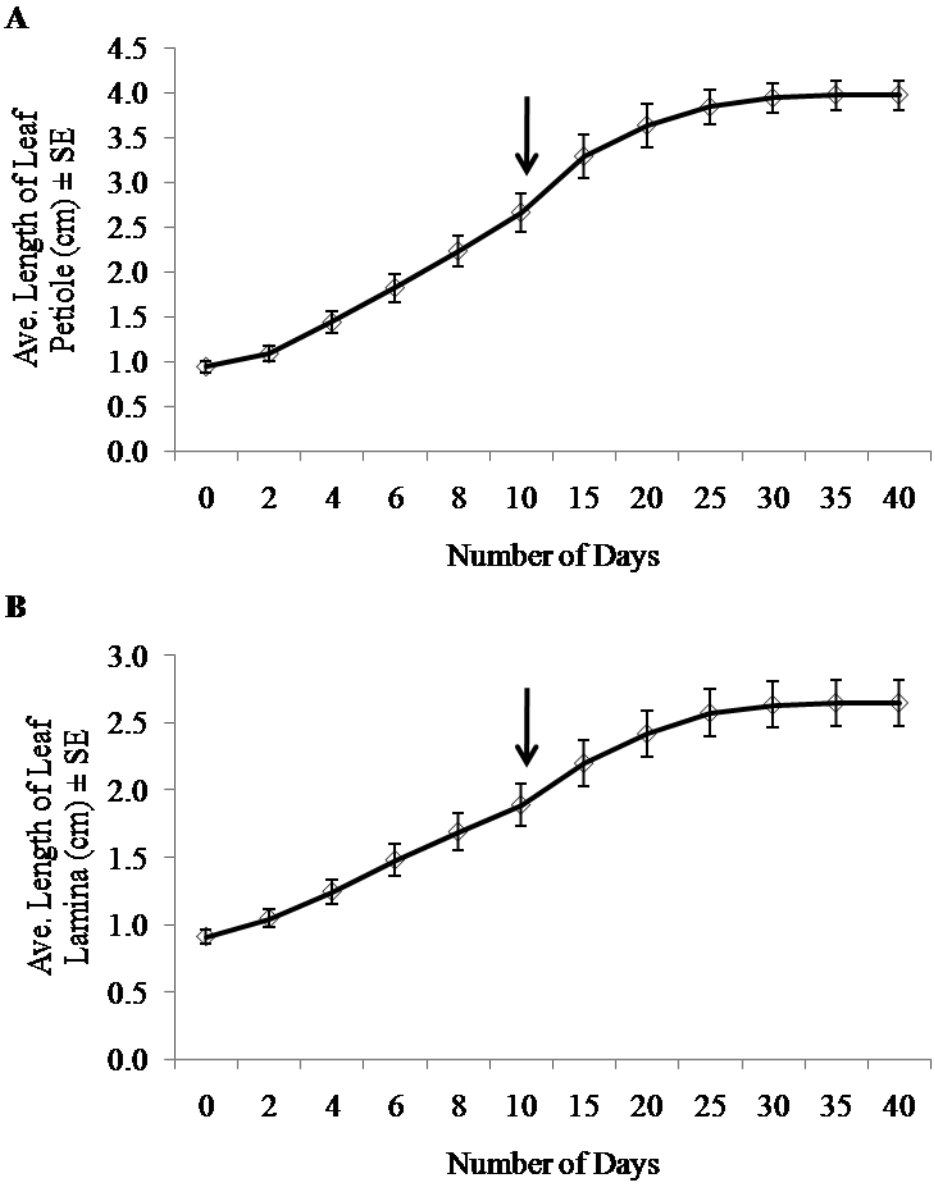

Fig 5.9. Growth curves of leaf petioles and laminas of tissue-cultured *A. andraeanum* cv 'Red Hot' plantlets (n = 25). A: Leaf petiole. B: Leaf lamina. Black arrows: Leaf opened.

5.3.4 Endopolyploidy analyses of greenhouse-grown plants and tissue-cultured plantlets

In the leaf lamina, petiole and root tissues of greenhouse-grown *A. andraeanum* cv 'Red Hot' plants analysed, only nuclei with 2C and 4C DNA content were detected (Table 5.2). Over 90 % of the nuclei in these tissues contained 2C DNA content. The 4C nuclei in these tissues accounted for a mean frequency of 0.87 to 8.88 % of the total population of nuclei. There was no significant difference in the endopolyploidy patterns of the leaf lamina, petiole and root tissues (Table 5.2).

The floral organs of *A. andraeanum* cv 'Red Hot' plants consist of spathes and spadices (see Fig 2.4). From 10 days before to 30 days after anthesis, no significant changes in the endopolyploidy patterns of the floral organs were observed (Table 5.2). The spathes were found to contain over 95 % of 2C nuclei (Table 5.2). Compared to the leaf petiole, lamina and root tissues, no significant difference in the mean frequencies of 2C and 4C nuclei in the spathes was observed (Table 5.2).

The spadices contained nuclei of up to 8C DNA content (Table 5.2). For the spadices at 10 days before and 30 days after anthesis, the mean frequencies of 2C nuclei ranged from 58.24 to 75.16 %, while the mean frequencies of 8C nuclei ranged from 5.25 to 11.71 % (Table 5.2). In addition, the spadices were found to contain less 2C nuclei than that in the leaf petioles and laminas, roots and spathes (Table 5.2).

For tissue-cultured plantlets, only nuclei with 2C and 4C DNA content were detected in the leaf petiole, lamina and root tissues (Table 5.3). Over 85 % of the nuclei in these tissues contained 2C DNA content and the mean frequency of the 4C nuclei ranged from 2.93 to 11.48 % (Table 5.3).

Table 5.2. Mean proportion of nuclei of various DNA contents (C-values) from different parts of greenhouse-grown *A. andraeanum* cv 'Red Hot' plants during development. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Type of Organ	Parts / Floral Stage	Mean Proportion of Nuclei (%) ± SE		
		2C	4C	8C
Leaf				
Unopened	Tip	96.10 ± 2.46^{ac}	3.90 ± 2.46^{a}	0.00 ± 0.00^{a}
	Base	97.61 ± 2.39^{a}	2.39 ± 2.39^{a}	0.00 ± 0.00^{a}
Fully Expanded	Tip	97.34 ± 2.66^{a}	2.66 ± 2.66^{a}	0.00 ± 0.00^{a}
	Base	99.13 ± 0.87^{a}	0.87 ± 0.87^{a}	0.00 ± 0.00^{a}
Petiole of				
Unopened Leaf	Whole	94.99 ± 2.89^{ac}	5.01 ± 2.89^{a}	0.00 ± 0.00^{a}
Fully Expanded Leaf	Whole	91.12 ± 5.85^{ac}	8.88 ± 5.85^{a}	0.00 ± 0.00^{a}
Roots				
2-cm	Whole	94.56 ± 2.63^{ac}	5.44 ± 2.63^{a}	0.00 ± 0.00^{a}
8-cm	Whole	95.22 ± 3.50^{ac}	4.78 ± 3.50^{a}	0.00 ± 0.00^{a}
Flower				
Spadix	10 Days Before	58.24 ± 3.59^{b}	36.51 ± 4.29^{b}	5.25 ± 1.93^{ab}
Spathe	Anthesis	99.11 ± 0.80^{a}	0.89 ± 0.80^{a}	0.00 ± 0.00^{a}
Spadix	30 Days After	75.16 ± 5.35^{bc}	13.13 ± 1.78^{ab}	11.71 ± 5.30^{b}
Spathe	Anthesis	97.91 ± 1.87^{a}	2.09 ± 1.87^{a}	0.00 ± 0.00^{a}

Table 5.3. Mean proportion of nuclei of various DNA contents (C-values) from different parts of tissue-cultured *A. andraeanum* cv 'Red Hot' plantlets during development. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Type of Organ	Parts	Mean Proportion of Nuclei (%) ± SE	
		2C	4C
Leaf			
Unopended	Tip	91.27 ± 4.14[a]	8.73 ± 4.14[a]
	Base	93.85 ± 2.09[a]	6.15 ± 2.09[a]
Fully Expanded	Tip	97.07 ± 2.93[a]	2.93 ± 2.93[a]
	Base	88.52 ± 1.20[a]	11.48±1.20[a]
Petiole of			
Unopened Leaf	Tip	89.69 ± 3.69[a]	10.3 ±3.69[a]
Fully Expanded Leaf	Tip	96.57 ± 3.43[a]	3.43 ± 3.43[a]
Roots			
8-cm	Whole	93.48 ± 4.17[a]	6.52 ± 4.17[a]

5.3.5 Effects of BA and 2,4-D on endopolyploidy in the callus tissues

Callus tissues of *A. andraeanum* cv 'Red Hot' were cultured in media containing different concentrations of BA or 2,4-D for 6 weeks. In the callus tissues cultured in media containing BA for 6 weeks, over 90 % of the nuclei in these callus tissues contained 2C DNA content, while the rest were 4C nuclei (Table 5.4). No significant difference was observed in the ploidy variations in the callus tissues cultured in basal medium and media containing different concentrations of BA (Table 5.4).

Similar results were obtained in callus tissues cultured in media containing 2,4-D. Only nuclei with 2C (over 90 %) and 4C (less than 10 %) DNA content were detected (Table 5.5).

Table 5.4. Effect of BA on the pattern of endopolyploidy in the callus tissues of *A. andraeanum* cv 'Red Hot' after 6 weeks of culturing in media containing BA, based on flow cytometric analyses of 10 000 nuclei per replicate. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Concentration of BA (μM)	Mean Proportion of Nuclei (%) ± SE	
	2C	4C
0.0	93.54 ± 3.44[a]	6.46 ± 3.44[a]
4.44	95.76 ± 2.13[a]	4.24 ± 2.13[a]
11.1	95.79 ± 2.14[a]	4.21 ± 2.14[a]
22.2	96.14 ± 2.01[a]	3.86 ± 2.01[a]
44.4	97.14 ± 2.86[a]	2.86 ± 2.86[a]

Table 5.5. Effect of 2,4-D on the pattern of endopolyploidy in the callus tissues of *A. andraeanum* cv 'Red Hot' after 6 weeks of culturing in media containing 2,4-D, based on flow cytometric analyses of 10 000 nuclei per replicate. Different letters following the standard error within the same column indicate significant difference based on Tukey's test at 95 % confidence interval.

Concentration of 2,4-D (μM)	Mean Proportion of Nuclei (%) ± SE	
	2C	4C
0.0	92.76 ± 3.67[a]	7.27 ± 3.67[a]
1.13	99.11 ± 2.66[a]	0.89 ± 2.66[a]
2.26	91.30 ± 1.21[a]	8.70 ± 1.21[a]
4.52	98.21 ± 1.44[a]	1.79 ± 1.44[a]
9.05	96.83 ± 1.51[a]	3.17 ± 1.51[a]

5.4 Discussion

5.4.1 Tissue culture

Effects of auxins on callus induction

Induction of callus or somatic embryos often involved combinations of varying concentrations of auxins and cytokinins. For instance in *Citrus mitis* cotyledon explants, medium containing auxin promoted callus and root formation, while high concentration of BA and low concentration of auxin resulted in shoot production (Sim et al. 1989). In the present study, concentration of BA in the medium was kept constant, while the concentration and the type of auxin varied. Here, using 4.44 µM BA and 2.26 µM 2,4-D or 5.71 µM IAA in culture media was optimal in inducing callus in leaf laminas of greenhouse-grown *A. andraeanum* cv 'Red Hot' (Table 5.1). However, using supra-optimal concentrations of 2,4-D or IAA inhibited callus induction. This is consistent with Kuehnle et al. (1992) who reported inhibition of embryogenic callus in the leaf lamina explants of other *A. andraeanum* cultivars (UH780, UH965 and UH1060) cultured in media supplemented with 2,4-D higher than 18.1 µM.

NAA was found to be ineffective in callus induction of leaf lamina explants in *A. andraeanum* cv 'Red Hot' (Table 5.1), although it has been reported to enhance callus formation in *A. andraeanum* cv 'Tinora Red' and 'Senator' (Martin et al. 2003). Such differences in response could be due to genetic variations between cultivars or differences in levels of endogenous auxin and cytokinin already present in the explants. This has been demonstrated in *Pennisetum Purpureum* where the embryogenic potential in leaf explants was found to be positively correlated with

endogenous plant growth regulators such as IAA and abscisic acid (Rajasekaran et al. 1987). As a result of variations among *A. andraeanum* cultivars, various media have to be formulated for callus induction in leaf lamina explants of different cultivars (Pierik et al. 1974, Kuehnle and Sugii 1991, Kuehnle et al. 1992, Teng 1997).

Effect of explant source on callus formation

2,4-D (2.26 µM) was found to be effective in callus induction for different types of explant obtained from the tissue-cultured plantlets (Fig 5.3). Explants from the distal end of leaf lamina were found to be least responsive to low concentration of 2,4-D (0 to 1.13 µM). The petiole and the proximal end of leaf lamina were found to be the better sources of explants for callus induction (Fig 5.3). This is consistent with Martin et al. (2003) who reported that explants from the proximal end of leaf lamina of *A. andraeanum* cv 'Tinora Red' and 'Senator' plants having higher callus induction and shoot regeneration potential than explants from the distal end. In *Sorghum bicolor*, callus was not observed in explants from mature, expanded leaf segments (Wernickle and Brettell 1980). The difference in the regeneration potential of the proximal and distal sections of leaf lamina explants of *A. andraeanum* cv 'Red Hot' plantlets could be due to differences in maturity of these parts as leaves reached maturity in a basipetal manner where distal end of the leaves matured first (Welander 1988). Another possible reason could be due to the difference in the concentration of endogenous plant growth regulators. This has been demonstrated in the leaf cultures of *P. purpureum* where high levels of endogenous IAA and abscisic acid were present at the proximal end of the leaves as compared to other regions and were positively correlated with their competence to form embryogenic callus (Rajasekaran et al. 1987).

Shoot regeneration from callus tissues

Organogenesis of callus culture involves shoot and root induction phases (Dunstan and Thorpe 1986). Auxin and cytokinin play important roles in plant morphogenesis and have great influence on the formation of roots and shoots (Dunstan and Thorpe 1986). The control of shoot and root formation in callus culture by varying the auxin to cytokinin ratio has been shown in *Medicago sativa* (Walker et al. 1979). In general, a low auxin-to-cytokinin ratio in the culture medium would induce shoot formation, while the reverse would promote root formation (Dunstan and Thorpe 1986). However, in leaf callus explants of *Brachycome dichromosomatica*, increasing auxin-to-cytokinin ratio in the culture medium initiated shoot formation (Gould 1979).

In the present study, callus tissues of *A. andraeanum* cv 'Red Hot' cultured on medium supplemented with 4.44 µM BA and 1.13 µM 2,4-D produced the highest number of shoots (21 shoots per callus explant) (Fig 5.4). This result demonstrated that shoot formation from callus explants was favoured in medium containing low auxin and high cytokinin concentration in the ratio of 1:4. In media containing 2,4-D of concentration higher than 1.13 µM, a decrease in the average number of shoots produced per callus explants was observed (Fig 5.4). This is consistent with Goh et al. (1994) who reported that high auxin levels in the culture medium inhibited the organogenetic process in mangosteen leaf cultures. This suggests that high concentration of auxin in the culture medium has inhibitory effect on shoot formation.

Shoot multiplication with cytokinin

Cytokinin alone in culture medium has been reported to induce shoot multiplication in plants such as *Houttuynia cordata* (Chakraborti et al. 2006), *Tinospora cordifolia*

(Raghu et al. 2006) and *Helianthus annuus* (Shin et al. 2000). Cytokinin such as BA was also reported to encourage shoot multiplication in some *A. andraeanum* cultivars (Martin et al. 2003). In the current study, the presence of cytokinin in the culture medium was found to induce shoot multiplication in *A. andraeanum* cv 'Red Hot' plantlets (Fig 5.5A). The optimum BA concentration for shoot multiplication of *A. andraeanum* cv 'Red Hot' plantlets was 11.1 µM. Furthermore, zeatin at 11.1 µM was the most effective cytokinin for shoot multiplication (Fig 5.7). The optimum cytokinin concentration for shoot multiplication of other anthurium cultivars, 'Tinora Red' and 'Senator', was 0.44 µM BA (Martin et al. 2003). In other plant, *H. cordata*, the optimum BA concentration for shoot multiplication was 8.87 µM (Chakraborti et al. 2006). These findings imply that the optimum concentration of cytokinin for shoot multiplication could be genotype dependent.

In *A. andraeanum* cv 'Red Hot', the presence of supra-optimal concentrations of BA (higher than 11.1 µM) in the culture medium decreased the average number of shoots produced per plantlet (Fig 5.5A). This is consistent with Martin et al. (2003) who reported the inhibition of shoot production in the plantlets of *A. andraeanum* cv 'Tinora Red' and 'Senator' cultured in media containing BA higher than 0.44 µM. In *H. cordata*, the number of shoots produced per plantlet was also reduced when cultured in media containing BA higher than 8.87 µM (Chakraborti et al. 2006).

In addition, it was observed that root production of *A. andraeanum* cv 'Red Hot' plantlets was significantly reduced with increasing concentrations of BA in the culture medium (Fig 5.5C). BA may possibly have inhibitory effect on root formation. Indeed, several studies using transgenic *Arabidopsis* and tobacco showed that severe root inhibition occurred in transgenic plantlets with high endogenous cytokinin (Li et al.

1992, Hewelt et al. 1994, Guo and Hu 2008). Inhibition of lateral root initiation was also reported in rice germinated in cytokinin (kinetin or zeatin) solution (Debi et al. 2005).

5.4.2 Growth patterns of greenhouse-grown plants and tissue-cultured plantlets

The growth patterns of leaves and flowers of *A. andraeanum* cv 'Red Hot' were investigated to determine the growth stages of the leaves and flowers to be used for flow cytometry analysis. The leaf petioles, laminas and flower peduncles of *A. andraeanum* cv 'Red Hot' exhibited an initial slow growth phase and an elongation phase, which was followed by a stationary phase where the organs matured and growth ceased (Figs 5.8A - C). However, the spathes of *A. andraeanum* cv 'Red Hot' did not exhibit similar growth pattern (Fig 5.8D). The development of the spathes of anthuriums began before the emergence of flowers from the leaf sheaths and was characterised by a double sigmoidal growth curve (Dai and Paull 1990). Hence, the spathes of *A. andraeanum* cv 'Red Hot' analysed could be at the second exponential growth phase when they emerged from the leaf sheaths.

In both greenhouse-grown plants and tissue-cultured plantlets of *A. andraeanum* cv 'Red Hot', the leaf laminas opened when the petioles reached stationary growth phase (Figs 5.8A and 5.9A). Similar finding was described in *A. andraeanum* Andre cv 'Kaumana' (Dai and Paull 1990). The young olive-brown leaves of *A. andraeanum* Andre cv 'Kaumana' were also reported to have net negative rate of photosynthesis, while mature fully expanded leaf (dark green) had the highest net positive rate of photosynthesis (Dai and Paull 1990). This suggests that the opening of leaves when maximum growth of petioles were reached could be an adaptation of the plant to

ensure that the leaves would receive maximum light once they were fully opened and their photosynthesis systems became fully developed.

5.4.3 Endopolyploidy in the tissues of greenhouse-grown plants and tissue-cultured plantlets

Endoreduplication was reported to be common in the reproductive organs of angiosperms (D'Amato 1984) and specific cell types or tissue (Nagl 1976, Galbraith et al. 1991, Carvalheira 2000). In the present study, multiploid cells were detected in the floral organ (spadix) of *A. andraeanum* cv 'Red Hot' plants (Table 5.2). In the flowers of cabbage (Kudo and Kimura 2001b), *Portulaca* (Mishiba and Mii 2000) and *D.* Chao Praya Smile (section 3.3.5), multiploid cells were also detected. The presence of multiploid cells in the reproductive tissues was suggested to be advantageous for their specialised function (Kudo and Kimura 2001b) as observed in the endosperm of maize kernels (Kowles et al. 1990) and suspensor cells of *Phaseolus* (Brodsky and Uryvaeva 1977). It was proposed that endoreduplication was required to increase transcriptional and metabolic activities that were essential for the function of specific cell types (D'Amato 1984). For instance, in the parenchyma cell of cotyledons of *P. sativum*, increased in polyploidy coincided with rapid increase in RNA and protein synthesis (Scharpe and Van Parijs 1973). In the development of maize endosperm, the extent of endoreduplication in the endosperm was positively correlated with the increase in storage proteins (zein) and starch (Lur and Setter 1993). Therefore, the higher ploidy level in the spadices of *A. andraeanum* cv 'Red Hot' may be required for their function as a reproductive organ.

On the other hand, endopolyploidy was not observed in most of the tissues of *A. andraeanum* cv 'Red Hot'. Leaf lamina, petiole and root tissues of both greenhouse-

grown plants and tissue-cultured plantlets consisted of majority 2C nuclei and some 4C nuclei (Tables 5.2 and 5.3). The low percentage of 4C nuclei (0.87 to 11.48 %) (Tables 5.2 and 5.3) could be due to some of the 2C nuclei that have entered or are arrested at the G2 phase of cell cycle. At the G2 phase of cell cycle, the amount of DNA in the nuclei will double (4C) (Doonan 2005).

The occurrence of minimal ploidy variations in the leaf lamina, petiole, root and spathe tissues was not unique to *A. andraeanum* cv 'Red Hot'. Endopolyploidy was also not observed in some members of the Aroid family (*Philodendron andreanum*, *Monstera deliciosa*, *Scindapsus aureus* (Olszewska and Osiecka 1982) and *A. scherzerianum* (Geier 1988)). In other plant such as *Helianthus*, polyploid cells were not found in the roots, cotyledons, stems, leaves, sepals, petals, pistils and stamens (Evans and Van't Hof 1975). These observations suggest that endopolyploidy is not an essential requirement for cell differention in all plants.

5.4.4 Effects of BA and 2,4-D on endopolyploidy in the callus tissues

Callus tissues are useful for genetic transformation, protoplast isolation and fusion (Ault and Siqueira 2008). For genetic transformation and micro-propagation, plants regenerated from the callus cultures have to be genetically stable. However, organogenesis from callus cultures holds the potential risk of somaclonal variation (Larkin and Scowcroft 1981). One of the possible factors affecting somaclonal variation is the occurrence of polyploid cells in the cultures (Gould 1986). Polyploid and aneuploid cells often occurred in callus and suspension cell cultures (Swedlund and Vasil 1985). The extent of polyploidy in callus and suspension cell cultures could be affected by the age of culture (Swedlund and Vasil 1985). In the *Pennisetum americanum*, 96 % of the cells in the calli induced with MS medium supplemented

with 11.3 µM 2,4-D was diploid. However, after 6 months of culturing in the same medium, the percentage of polyploid and aneuploid cells in the callus cultures increased (Swedlund and Vasil 1985).

Another factor affecting the occurrence of polyploid cells in cultures is the use of plant growth regulators in culture medium (Gould 1986). For instance, in the suspension cell cultures of the orchid *Doritaenopsis*, culture medium containing BA or thidiazuron reduced endoreduplication in the cells (Mishiba et al. 2001), while in culture medium supplemented with 2,4-D or picloram, an increase in endoreduplication in the cells was resulted (Mishiba et al. 2001).

In contrast, the addition of BA or 2,4-D to the culture medium has no effect on endopolyploidy in the callus tissues of *A. andraeanum* cv 'Red Hot' (Tables 5.4 and 5.5). Moreover, polyploid cells were not observed in the callus tissue cultured in plant-grown-regulator-free medium for 6 weeks (Tables 5.4 and 5.5). These results indicated that the callus tissues of *A. andraeanum* cv 'Red Hot' were genetically stable. This is not unique to *A. andraeanum* cv 'Red Hot'. For instance, in alfalfa, callus cultures induced using high concentration of 2,4-D were also found to be karyologically stable (Binarova and Dolezel 1988).

As aforementioned, nuclear stability was observed in the leaves and petioles of tissue-cultured plantlets of *A. andraeanum* cv 'Red Hot' (section 5.4.3), which were the explant sources for callus induction. Therefore, the nuclear stability observed in the callus tissues could be attributed to the genetic stability in the explant source. In addition, callus cultures induced using medium containing 4.44 µM BA and 2.26 µM 2,4-D was observed to be genetically stable. This genetic stability suggests that callus cultures of *A. andraeanum* cv 'Red Hot' could be used for micro-propagation, genetic

transformation and protoplast isolation and fusion with minimal risk of somaclonal variation.

Chapter 6 Concluding remarks

Two horticulturally important plants, an orchid hybrid (*D.* Chao Praya Smile) and an anthurium hybrid (*A. andraeanum* cv 'Red Hot'), were used in this study. *D.* Chao Praya Smile showed high degree of endopolyploidy in the tissues, while *A. andraeanum* cv 'Red Hot' was relatively stable. The maximal C-values found in the nuclei of different parts of greenhouse-grown and aseptically-grown *D.* Chao Praya Smile plants and *A. andraeanum* cv 'Red Hot' plants are summarised in Figs 6.1 - 6.3.

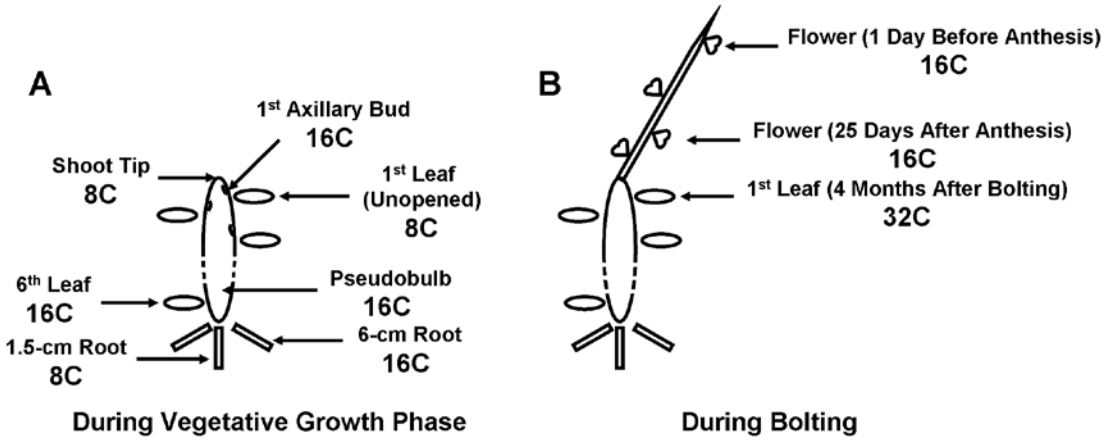

Fig 6.1. Maximal C-values found in the nuclei of different parts of greenhouse-grown *D.* Chao Praya Smile plants.

A: Plants during vegetative growth phase.

B: Plants flowered for the first time.

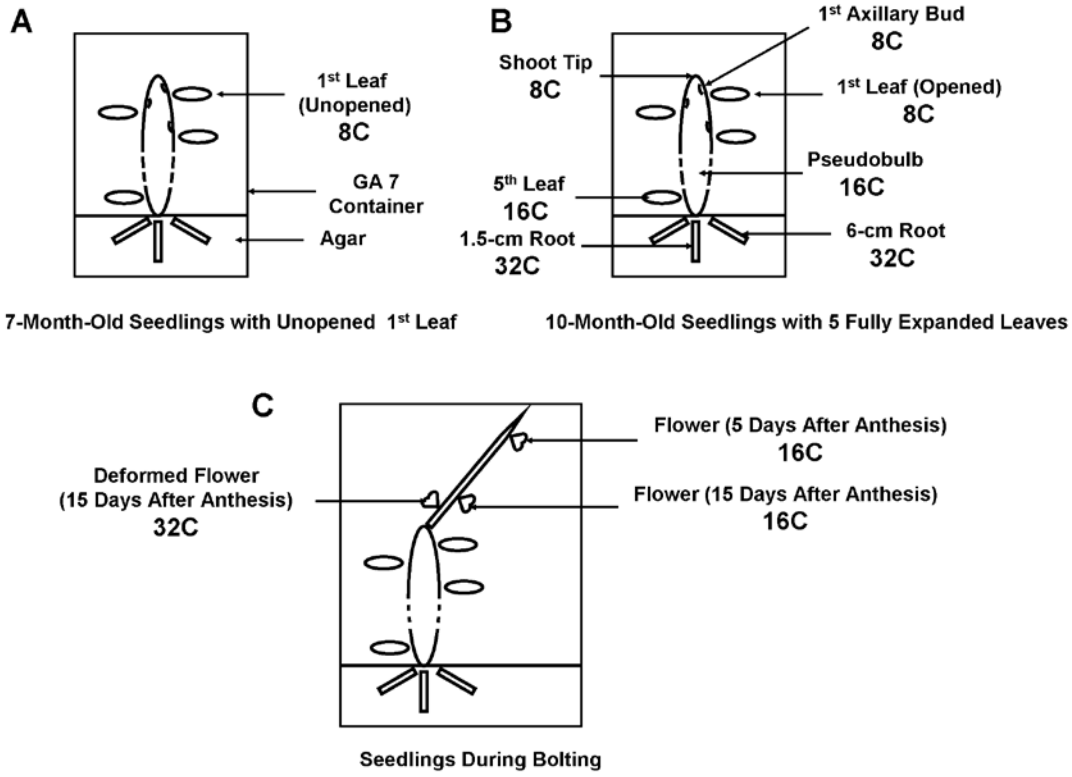

Fig 6.2. Maximal C-values found in the nuclei of different parts of aseptically-grown *D.* Chao Praya Smile seedlings.

A: Seven-month-old seedlings with unopened 1st leaf.

B: Ten-month-old seedlings with five fully expanded leaves.

C: Seedlings during bolting.

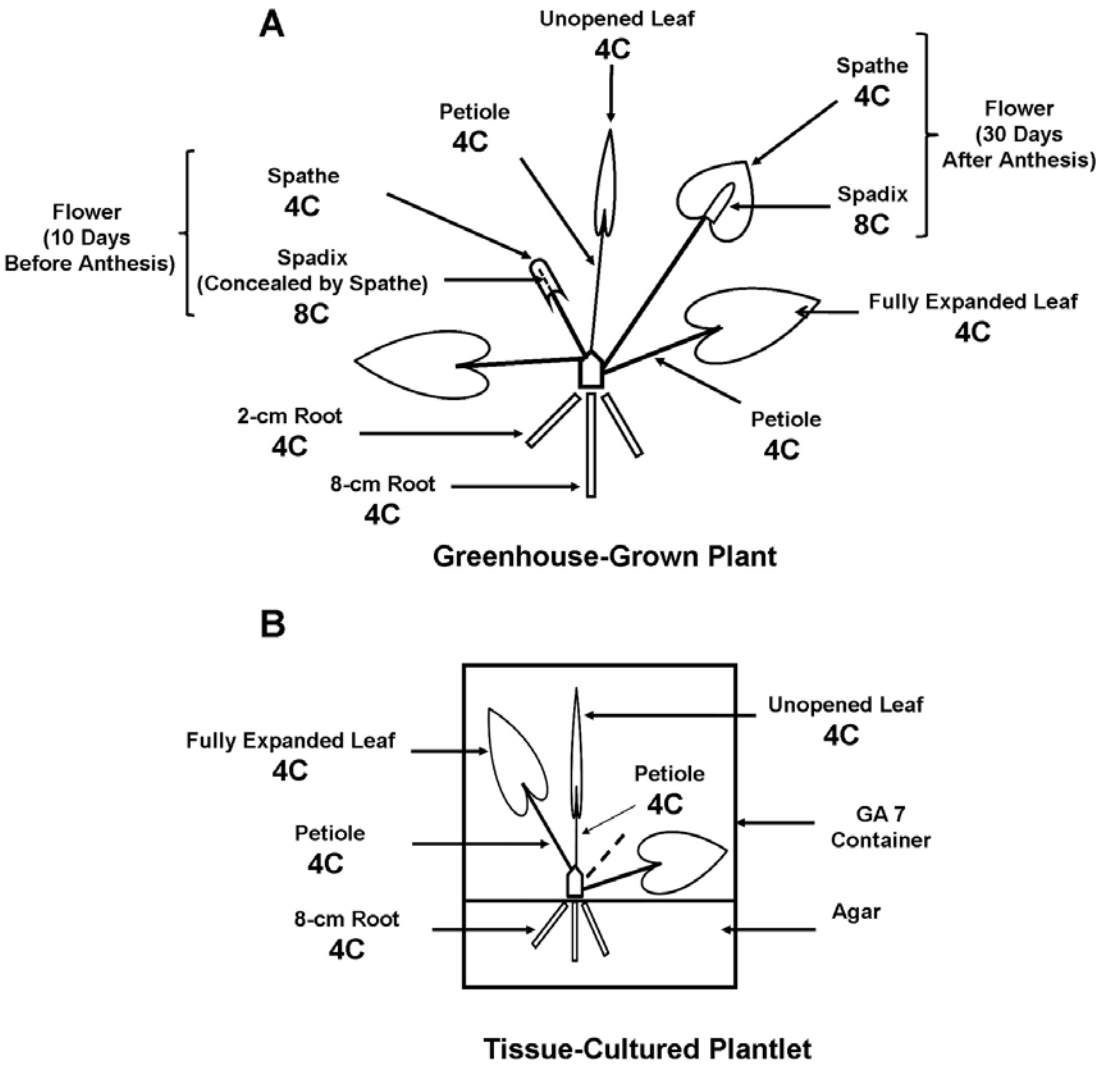

Fig 6.3. Maximal C-values found in the nuclei of different parts of greenhouse-grown plants and tissue-cultured plantlets of *A. andraeanum* cv 'Red Hot'.

A: Greenhouse-grown plants.

B: Tissue-cultured plantlets.

Multiploid cells were found in majority of the tissues with varying patterns in both greenhouse-grown plants and aseptically-grown seedlings of *D.* Chao Praya Smile (Figs 6.1 and 6.2). This indicates the presence of systemic control of endopolyploidy in *D.* Chao Praya Smile plants. Endoreduplication in the protocorms, leaves and roots were found to be developmentally regulated. These findings are in line with the observations in *V.* Miss Joaquim (Lim and Loh 2003) and *S. plicata* (Yang and Loh 2004). This current study also showed that the fully expanded leaves of greenhouse-grown plants exhibited higher ploidy variation than that of aseptically-grown seedlings of *D.* Chao Praya Smile. The roots of aseptically-grown *D.* Chao Praya Smile seedlings were found to exhibit higher ploidy level as compared to that from the greenhouse-grown plants (Figs 6.1A and 6.2B). These results imply that the endoreduplication process in *D.* Chao Praya Smile is affected by tissue-culture conditions such as light and the presence of plant growth regulators. The addition of plant growth regulators such as BA, 2,4-D, GA_3, TIBA or PAC to the culture medium was found to affect endopolyploidy in the protocorm cultures of *D.* Chao Praya Smile. These suggest that the use of these plant growth regulators during micro-propagation of *Dendrobium* could possibly affect endoreduplication in the cells during tissue culture. This in turn may affect the degree of somaclonal variation exhibited among the regenerants.

In both *D.* Chao Praya Smile and *A. andraeanum* cv 'Red Hot', endopolyploidy was detected in the reproductive tissues and was found not to be developmentally regulated. In *D.* Chao Praya Smile, nuclei of up to 16C DNA content were detected in the column of flowers (Figs 6.1A and 6.2B). In *A. andraeanum* cv 'Red Hot', nuclei of up to 8C DNA content were found in the spadices (Fig 6.3A). The presence of

multiploid cells in the reproductive tissues was suggested to be advantageous for their specialised function (Kudo and Kimura 2001b). D'Amato (1984) also proposed that endoreduplication was required to increase transcriptional and metabolic activities that were essential for the function of specific cell types. In the development of maize endosperm, the extent of endoreduplication in the endosperm was positively correlated with the increase in storage proteins (zein) and starch (Lur and Setter 1993). In *P. sativum*, increased in polyploidy in the parenchyma cell of cotyledons coincided with rapid increase in RNA and protein synthesis (Scharpe and Van Parijs 1973). Therefore, the higher degree of endopolyploidy in the reproductive tissues of *D.* Chao Praya Smile and *A. andraeanum* cv 'Red Hot' could possibly serve to increase their RNA and protein synthesis for their function as reproductive organs.

Unlike *D.* Chao Praya Smile, the nuclei in the tissues of *A. andraeanum* cv 'Red Hot' plants were found to be relatively stable and had minimum ploidy variations. In both greenhouse-grown plants and tissue-cultured plantlets of *A. andraeanum* cv 'Red Hot', endoreduplication was not observed in the tissues of leaves, petioles, roots and spathes. The maximal C-value detected in these tissues were 4C (Fig 6.3). Endopolyploidy was also not observed in the callus tissues. Addition of 2,4-D or BA to the culture medium did not affect endopolyploidy variation in the callus tissues.

In previous studies, endoreduplication was not observed in some members of the Aroid family, namely *Philodendron andreanum*, *Monstera deliciosa*, *Scindapsus aureus* (Olszewska and Osiecka 1982) and *A. scherzerianum* (Geier 1988). In the present study, *A. andraeanum* cv 'Red Hot', which is an Aroid, also exhibited minimal ploidy variations. In contrast, endoreduplication is common in the Orchidaceae family and was observed in orchids such as *V. sanderiana* (Alvarez 1968), *Doritaenopsis*

(Mishiba et al. 2001), *Cymbidium* (Fukai et al. 2002), *V.* Miss Joaquim (Lim and Loh 2003), *S. plicata* (Yang and Loh 2004) and *D.* Chao Praya Smile (sections 3.3 and 3.4). These results suggest that the occurrence of endopolyploidy is not ubiquitous among plant families.

The findings on endopolyploidy in this study have provided a better insight on the relation between endopolyploidy and somaclonal variation. Somaclonal variation is common in micro-propagated plants (Lee and Phillips 1988). The degree of endoreduplication in the original explants has been suggested to affect the extent of somaclonal variation in cultures (Evans 1989). In cucumber, the occurrence of multiploid cells was found to be common in the leaves (Gilissen et al. 1993) and the degree of somaclonal variation was reported to be related to the type of explants used (Plader et al. 1998, Ladyzynski et al. 2002). This implies that the degree of ploidy variation in the initial explants might affect the extent of somaclonal variation in the regenerants. For instance, in tomato, the existence of multiploid cells in the original explants was found to be positively correlated with the frequency of variants observed in the regenerants (Van den Bulk et al. 1990). In the current study, multiploid cells were not detected in the initial explant sources (young leaf petioles and laminas), callus tissues and the regenerants (sections 5.4.3 and 5.4.4) of *A. andraeanum* cv 'Red Hot'. The absence of multiploid cells in the initial explant sources could possibly result in the nuclei stability of the regenerants. However, the results from the present study on *A. andraeanum* cv 'Red Hot' were insufficient to suggest that pre-existing genetic differences in somatic cells of the initial explants used was a possible cause of somaclonal variation during micro-propagation as hypothesised by Evans (1989).

Further investigations to validate this speculation in *A. andraeanum* cv 'Red Hot' are required.

In orchids, there is also little direct evidence to correlate such variations in the regenerants with variations in the karyotype of the parental cells. This study has provided some insight in the cellular ploidy levels in different plant parts of *D.* Chao Praya Smile and *A. andraeanum* cv 'Red Hot' and enhanced the understanding on the nature of the various explants used for micro-propagation. Knowledge obtained from this study would facilitate future work on elucidating the relationship between somaclonal variation and endopolyploidy in these two plants.

Chapter 7 References

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