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# SOMATIC EMBRYOGENESIS AND TRANSFORMATION IN TARO (*Colocasia esculenta* var. *esculenta*)

**by**

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**A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy**

**School of Biological, Chemical and Environmental Sciences**

**Faculty of Science and Technology**

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**August, 2008**

# Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet the requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

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# Certification

This is to certify that this thesis entitled "Somatic Embryogenesis and Transformation in Taro (*Colocasia esculenta* var. *esculenta*)" submitted for the Doctor of Philosophy (PhD) in the subject area of Molecular Biology and Biotechnology to the University of the South Pacific, is a bonafide research work carried out by Mr. Pradeep Chand Deo under our supervision and guidance. No part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation has been duly acknowledged.

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# Abstract

Taro (*Colocasia esculenta* L. Schott) is an important crop worldwide but is of particular significance in many Pacific Island countries where it forms part of the staple diet and serves as an export commodity. Escalating pests and disease problems are jeopardizing taro production with serious implications to food security and trade. One possible solution to these problems is the generation of resistant cultivars using genetic engineering strategies. Therefore, the aim of this study was to develop an efficient regeneration system for *Colocasia esculenta* var. *esculenta*, the preferred taro variety in Pacific Island countries, via somatic embryogenesis and to investigate the use of this system for developing an efficient taro transformation protocol.

Embryogenic callus was initiated in taro cultivars, CPUK, CK-07 and THA-07, by culturing corm explants on half-strength MS medium containing 2.0 mg/L 2,4-D for 20 days followed by subculture to the same medium containing 1.0 mg/L TDZ. Callus production was significantly affected by the explant type, light/dark culture, 2,4-D and TDZ concentration, MS medium strength, the duration of exposure of the explants to 2,4-D and the genotype. Once generated, callus was proliferated on agar-solidified medium containing 1.0 mg/L TDZ, 0.5 mg/L 2,4-D and 800 mg/L glutamine or in liquid culture in the same medium but with reduced glutamine (100 mg/L). Regeneration from callus growing on solid medium was achieved in a single step on hormone free half-strength MS medium whereas a reduction in the concentrations of 2,4-D and TDZ to one-tenth of that used for proliferation, increasing the concentration of sucrose to 40-50 g/L and adding biotin (1.0 mg/L), improved somatic embryo formation significantly from suspension-derived cells. Incorporation of BAP and IAA enhanced maturation and germination frequency. Somatic embryos formed at a rate of approximately 500-3000 per mL settled cell volume and 80-100 per gram solid media-derived callus with embryos converting to plantlets at a rate of approximately 65 %.

Transformation protocols were developed with both *Agrobacterium tumefaciens* (AGL 1 strain) and microprojectile bombardment using the plasmid pART-TEST 7 containing the

*gfp* reporter and *npt* II selectable marker genes. Embryogenic suspension cells of CPUK and THA-07 were placed 7.5 cm from the point of particle discharge and bombarded with gold microprojectiles at a helium pressure of 550 kPa and vacuum of 85 kPa. Three days post-bombardment, numerous green fluorescing foci were observed. The transformation efficiency was enhanced by incubating cells on osmoticum (0.2 M mannitol and 0.2 M sorbitol) for two hours prior to and post-bombardment. Fluorometric assay of transient *uid*A (GUS) reporter gene expression in suspension cells demonstrated that the maize polyubiquitin-1 (*Ubi*-1) promoter activity was 1.4-fold higher than the cauliflower mosaic virus (CaMV 35S) and 23-fold higher than taro bacilliform virus (TaBV-600) promoters. Suspension cells were mixed with *A. tumefaciens* suspension, centrifuged then co-cultivated at 23 $^{o}$C for three days in darkness. Bacterial cell density, acetosyringone, co-cultivation media, co-cultivation temperature, heat shock of plant cells and genotype significantly influenced transformation efficiency. Regeneration was monitored from single cells to whole plants using *gfp* reporter gene expression. Transformed cells were selected on kanamycin (150 mg/L) with timentin (200 mg/L) also included to control *Agrobacterium*. Putatively stably transformed embryos were obtained using both methods with an efficiency of ~200 per mL settled cell volume using microprojectile bombardment and ~17 per mL settled cell volume using *Agrobacterium*. Molecular characterization of these transformants was not possible, as embryos/plants had not reached a sufficient size within the timeframe of this project. However, analyses to verify transgene presence and integration will be done once the plants are sufficiently developed.

The development of somatic embryogenesis and transformation protocols in this study provides an opportunity to significantly increase taro production in the Pacific through the generation of transgenic taro with traits not possible to introduce through conventional breeding.

**Key words:** Taro, *Colocasia esculenta* var. *esculenta*, transformation, microprojectile bombardment, *Agrobacterium tumefaciens*, somatic embryogenesis, embryogenic cell suspension, promoter.

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# List of Abbreviations

| | | |
|---|---|---|
| ABA | = | abscisic acid |
| AFLP | = | amplified fragment length polymorphism |
| APES | = | 3-aminopropyltriethoxysilane |
| ATP | = | adenosine triphosphate |
| BAP | = | 6-benzylaminopurine |
| bp | = | base pairs |
| CH | = | casein hydrolysate |
| CE | = | corn extract |
| cm | = | centimetre(s) |
| DNA | = | deoxyribonucleic acid |
| 2,4-D | = | 2,4-dichlorophenoxyacetic acid |
| GA | = | gibberellic acid |
| g | = | gram(s) |
| *gfp* | = | gene encoding GFP |
| GFP | = | green fluorescent protein |
| Gln | = | glutamine |
| GUS | = | β-glucuronidase |
| HCl | = | hydrochloric acid |
| IAA | = | indole-3-acetic acid |
| IBA | = | indole-3-butyric acid |
| kb | = | kilo base pairs |
| Kinetin | = | 6-furfurylaminopurine |
| kPa | = | kilopascal(s) |
| M | = | molar (moles per litre) |
| mg/L | = | milligrams per litre |
| ml/L | = | millilitres per litre |
| m | = | metre(s) |
| mL | = | millilitre(s) |
| MS | = | Murashige and Skoog medium (1962) |
| MU | = | 4-methylumbelliferone |

| | | |
|---|---|---|
| NAA | = | naphthalene acetic acid |
| NaOH | = | sodium hydroxide |
| *npt* II | = | gene encoding NPT II |
| NPT II | = | neomycin phosphotransferase |
| OD | = | optical density |
| PAE | = | papaya extract |
| PE | = | potato extract |
| PEG-4000 | = | polyethyleneglycol-4000 |
| PEM | = | pro-embryogenic mass |
| ρmoles | = | pico moles |
| rpm | = | revolutions per minute |
| SCV | = | settled cell volume |
| SE | = | somatic embryo |
| SEM | = | standard error of the mean |
| SPSS | = | statistical package for social sciences |
| SSR | = | simple sequence repeats |
| TDZ | = | thidiazuron |
| TE | = | taro corm extract |
| 2,4,5-T | = | 2,4,5-trichlorophenoxyacetic acid |
| µg | = | microgram |
| µL | = | microlitre(s) |
| µm | = | micrometre |
| µM | = | micromolar |
| Zeatin | = | 6-(4-hydroxy-3-methyl-2-butenylamino) purine |

# CHAPTER 1

## Introduction

Taro (*Colocasia esculenta* var. *esculenta*) is an important tropical crop cultivated throughout Oceania, parts of Africa, Asia and the Caribbean for its fleshy starchy corms. It has evolved from a subsistence crop into a multimillion-dollar industry, playing a significant role in the economy of some Pacific Island countries.

### *1.1 Description of Scientific Problem Investigated*

Like other crops, taro production in the Pacific is hampered by many pests and diseases. The development of resistant taro varieties via conventional breeding is a difficult and lengthy process. However, plant tissue culture and biotechnology techniques can be utilized to develop resistant taro varieties within a relatively short timeframe. Somatic embryogenesis and gene transformation have become important tools in plant biotechnology and protocols for both technologies have been developed in a number of important agronomic crops. Generating large numbers of transgenic taro in order to screen for the new trait while retaining the parent plants desirable qualities demands an efficient regeneration system such as somatic embryogenesis.

Despite the establishment of *in vitro* propagation in taro, there are no reports of indirect somatic embryogenesis, either via embryogenic callus or suspension cell cultures, in *Colocasia esculenta* var. *esculenta*, the preferred taro variety in the Pacific. The unavailability of an efficient regeneration system and transformation-competent target tissues are the major obstacles to the development of an efficient transformation system for *esculenta* taro varieties. Thus, there is an urgent need to have available an efficient regeneration and transformation protocol for *Colocasia esculenta* var. *esculenta*, to address pest and disease pressures. Developing somatic embryogenesis and transformation systems for taro could also boost the production of

good quality taro for Pacific Island countries where taro has been a major subsistence and export commodity.

## *1.2 Overall Objectives of the Study*

The aim of this research project was to develop a somatic embryogenesis system as a mass propagation system for taro and to investigate the use of this system in the development of efficient transformation systems using microprojectile bombardment and/or *Agrobacterium tumefaciens*-mediated transformation.

## *1.3 Specific Aims of the Study*

The specific aims of this research were to: (i) develop and optimize a protocol for the induction of embryogenic callus using different explant tissues, (ii) develop and optimize a system for the proliferation of embryogenic tissues either via callus, secondary somatic embryogenesis or suspension cell cultures, (iii) determine whether somatic embryogenesis in taro is genotype dependent, (iv) determine the optimum culture conditions for the regeneration of taro somatic embryos into viable plantlets and (v) develop and optimize the protocol for genetic transformation in taro either via microprojectile bombardment or *Agrobacterium tumefaciens*-mediated transformation.

## *1.4 Account of Scientific Progress Linking the Scientific Chapters*

Before the commencement of this research project, there were no published methods on indirect somatic embryogenesis in *Colocasia esculenta* var. *esculenta*. Therefore, preliminary experiments (Chapter 4) were conducted to initiate embryogenic callus using Murashige and Skoog (MS) (1962) medium containing various combinations and concentrations of the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) with different explants. Incubating petiole explants from third leaf primordia on 2,4-D for various durations (3, 6, 9, 12 and 15 days) followed by transfer on TDZ showed potential for callus formation the frequency of

which improved when the petioles were substituted with corm sections as explants. Embryogenic callus formed in corm explants when the time of incubation on 2,4-D was increased to 20 days; hence, this protocol was further refined using corm explants. Various factors affecting the initiation of embryogenic callus in *Colocasia esculenta* var. *esculenta* were then studied and optimized. A factorial design was used to determine the most favourable combination and concentration of 2,4-D and TDZ required for embryogenic callus initiation. Once this was obtained, the optimal time of incubation of the explants on 2,4-D was studied using three incubation times (10, 20 and 40 days). The optimal conditions derived from these two experiments were then used to study the effect of the strength of MS salts, vitamins and taro genotype on somatic embryogenesis.

Having established the protocol for embryogenic callus initiation, the conditions required for callus proliferation, maintenance and subsequent regeneration of somatic embryos were studied (Chapter 5). A factorial design was again used to determine the most suitable combination and concentration of 2,4-D and TDZ required for embryogenic callus proliferation. Once determined, various concentrations of glutamine were used to improve the maintenance and longevity of embryogenic callus, resulting in the development of agar-solidified callus maintenance medium ($CMM_S$). An effective suspension cell culture medium ($CMM_L$) was subsequently developed based on this medium (minus agar), supplemented with glutamine and additional sucrose. The factors affecting the regeneration and maturation of somatic embryos from both culture methods were then examined and optimized, resulting in the development of an efficient regeneration system.

The final experimental chapter (Chapter 6) describes preliminary studies towards the development of a taro transformation protocol. Using the microprojectile bombardment approach, a much higher level of transient reporter gene (GFP) activity was observed in suspension cells compared to solid media-derived callus. Therefore, all transformation experiments were conducted using suspension cells as the target material. These cells were subsequently used to optimize bombardment conditions using transient reporter gene assays. Finally, the activity of three promoters,

cauliflower mosaic virus 35S promoter (CaMV 35S), maize polyubiquitin-1 gene (*Ubi*-1) and taro bacilliform virus promoter (TaBV-600), was assessed transiently in taro suspension cells by fluorometric analysis using the *uid* A (GUS) reporter gene. Following the development of a biolistic transformation protocol, *Agrobacterium tumefaciens*-mediated transformation was also examined. A protocol used for banana transformation was initially tested but this system was subsequently refined which resulted in a 4 to 5-fold increase in transient GFP expression. Additional parameters affecting the transformation efficiency were also studied. Taro suspension cells were then transformed with *gfp* reporter and *npt* II selectable marker gene, placed on selective medium and putatively transgenic taro plants were regenerated via embryogenesis.

# CHAPTER 2

# Literature Review

## *2.1 Introduction to Taro - A Monocotyledonous Tropical Root Crop Used in This Study*

Taro is an important staple food crop grown throughout many Pacific Island countries. In addition to contributing to sustained food security in the domestic market, it also brings in export earnings (Revill *et al.*, 2005). The plant can fit well into tree crop and agro forestry systems and some types are particularly well adapted to unfavourable land and soil conditions such as poor drainage. As such, taro is grown under intensive cultivation as a starch crop (Jianchu *et al.*, 2001).

### 2.1.1 Morphology, botany and genetics

Taro (*Colocasia esculenta*) is a herbaceous plant which grows to a height of 1-2m. The plant consists of a central corm lying just below the soil surface, with leaves growing from the apical bud at the top of the corm and roots grow from the lower portion. Cormels, daughter corms and runners grow laterally. The leaf is peltate and the root system is fibrous and restricted mainly to the top one metre of soil (Figure 2.1). The corm is a nutrient storage organ similar to yam or potato. The corm of *Colocasia esculenta* shares the following characteristics with food storage organs in carrot, sweet potato and manioc (Miyasaka, 1979):

- abundance of periderm.
- food storage in large thin walled parenchymatous cells.
- poorly developed vascular bundles that are few in number.
- presence of latex cells, mucilage cells and ergastic substances such as druses and raphides.

Taro belongs to the class of monocotyledons and family *Araceae* (Onwueme, 1978; Ivancic, 1992). The cultivated taro is classified as *Colocasia esculenta*, but the species is considered to be polymorphic (Purseglove, 1972).

There are eight recognized variants within *Colocasia esculenta*, of which two are commonly cultivated (O'Sullivan *et al.*, 1996): *C. esculenta (L.) Schott* var. *esculenta* and *C. esculenta (L.) Schott* var. *antiquorum. C. esculenta* var. *esculenta* possesses a large cylindrical central corm and very few cormels (Figure 2.2A); agronomically it is referred to as the 'dasheen' type of taro. In contrast, *C. esculenta* var. *antiquorum* has a small globular central corm with several relatively large cormels arising from the corm (Figure 2.2B); agronomically this variety is referred to as the 'eddoe' type of taro (Purseglove, 1972; Lebot and Aradhya, 1991). Most of the taro grown in the Asia Pacific region is of the dasheen type. Where taro is grown primarily for leaves, *C. esculenta* var. *antiquorum* is preferred (O'Sullivan *et al.*, 1996).

Chromosome numbers reported for taro from various regions include 2n = 22, 26, 28, 38 and 42 (Onwueme, 1978). The most commonly reported chromosome numbers are: diploids 2n = 28 and triploids 3n = 42 (Kuruvilla and Singh, 1981; Wang, 1983; Lebot and Aradhya, 1991; Lee, 1999). Furthermore, plants with 3n = 42 are referred to as *alowane* (male, large plant) and those of 2n = 28 are referred to as *alokine* (female, short plant) by Solomon Island farmers (Jackson *et al.*, 1977; Wang, 1983).

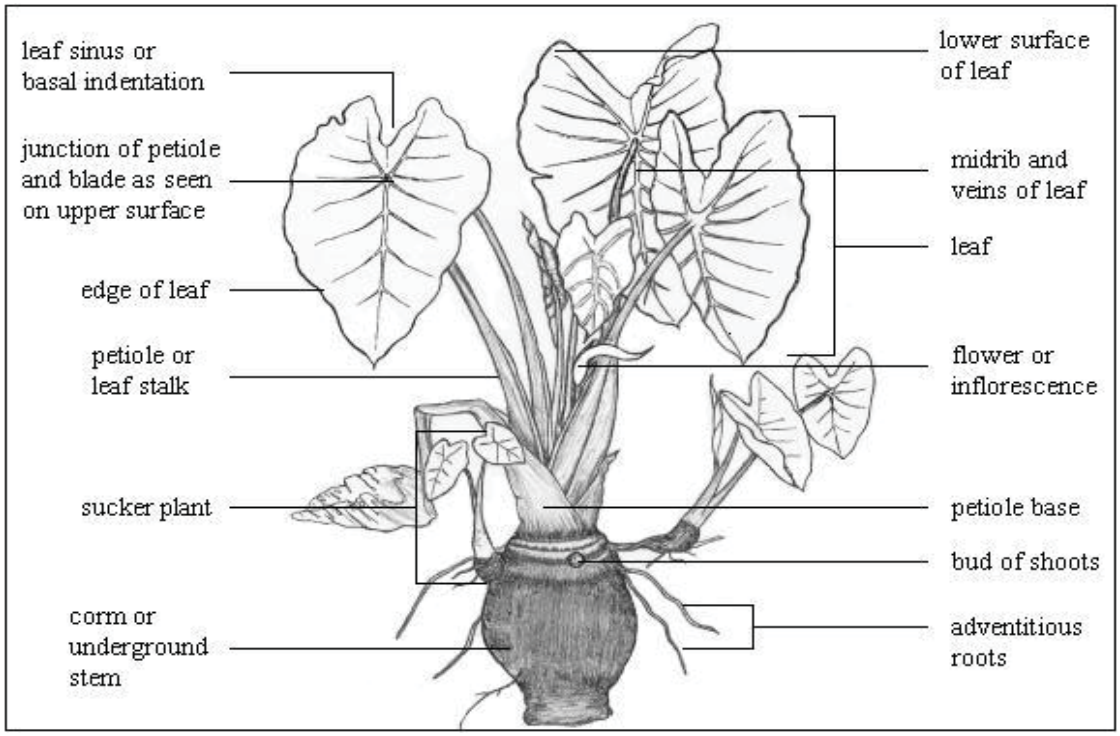


**Figure 2.1:** Diagram depicting parts of a taro plant (*C. esculenta* var. *esculenta*) (CTAHR, 1997)


**Figure 2.2:** The two major taro cultivars can be identified by corm structure.

*C. esculenta* var. *esculenta* (dasheen) has a large central corm (A), while *C. esculenta* var. *antiquorum* (eddoe) has a small central corm with multiple relatively large cormels.

### 2.1.2 Origin and distribution

Taro is believed to have originated in North Eastern India and Asia (Kuruvilla and Singh, 1981; Hanson and Imamuddin, 1983; Ivancic, 1992). From its centre of origin it spread eastward into Southeast Asia, Eastern Asia and the Pacific Islands; westward to Egypt and the Eastern Mediterranean and then southward and westward from there into East Africa and West Africa, from where it spread to the Caribbean and America (Onwueme, 1978; Lee, 1999). Taro is now grown in more than 65 countries worldwide (USDA, 2001). Using isozyme analysis, Lebot and Aradhya (1991), reported the existence of two gene pools for cultivated taro; one in Asia and the other in Pacific. Recent studies with SSR (Simple Sequence Repeats) markers (Noyer *et al.*, 2003) and AFLP (Amplified Fragment Length Polymorphism) markers (Kreike *et al.*, 2004) have confirmed the existence of these two distinct gene pools. This indicates that taro was domesticated in Asia as well as in the Pacific; therefore, it can now be considered as a native plant of the Pacific.

### 2.1.3 Nutrition

Nutritive values (Table 2.1 and Table 2.2) indicate that the taro corm is an excellent source of carbohydrate. It contains various carbohydrates, the majority being starch of which 17-28 % is amylose with the remainder amylopectin. The size of a taro starch grain is one-tenth that of potato and its digestibility has been estimated to be 98.8 %. Because of its ease of assimilation, it is suitable for persons with digestive problems. Taro is especially useful to those allergic to cereals and can be consumed by children who are sensitive to milk and, as such, taro flour is used in infant formulae and canned baby foods (Lee, 1999). Taro corm is low in fat and protein; however, the protein content is slightly higher than that of yam, cassava or sweet potato. The protein is rich in some essential amino acids, but is low in isoleucine, tryptophan and methionine (Onwueme, 1978). Taro leaves contain a higher level of protein than the corms and are also an excellent source of carotene, potassium, calcium, phosphorous, iron, riboflavin, thiamine, niacin, vitamin A, vitamin C and dietary fibre (Onwueme, 1978; Lambert, 1982; Hanson and Imamuddin, 1983; Bradbury and Holloway, 1988; Opara, 2001). They also contain greater amounts of vitamin B-complex than whole milk (Lee, 1999) and are higher in protein and other nutrients than tannia (new

cocoyam; *Xanthosoma sagittifolium*) the exception being oil. The fresh taro leaf lamina contains 80 % moisture while the petiole has 94 %.

**Table 2.1:** Nutritional information for taro

| Components | per 100g edible portion | | |
|---|---|---|---|
| | Corms | Leaves | Petioles |
| Energy (cal) | 85 | 69 | 19 |
| Moisture (%) | 77.5 | 79.6 | 93.8 |
| Protein (g) | 2.5 | 4.4 | 0.2 |
| Fat (g) | 0.2 | 1.8 | 0.2 |
| Carbohydrate (g) | 19 | 12.2 | 4.6 |
| Fibre (g) | 0.4 | 3.4 | 0.6 |
| Calcium (mg) | 32 | 268 | 57 |
| Phosphorous (mg) | 64 | 78 | 23 |
| Sodium (mg) | 7 | 11 | 5 |
| Potassium (mg) | 514 | 1237 | 367 |
| Iron (mg) | 0.8 | 4.3 | 1.4 |
| Vitamin (IU) | Trace | 20385 | 335 |
| Thiamine (mg) | 0.18 | 0.1 | 0.01 |
| Riboflavin (mg) | 0.04 | 0.33 | 0.02 |
| Niacin (mg) | 0.9 | 2 | 0.2 |
| Ascorbic acid (mg) | 10 | 142 | 0.8 |

(Department of Primary Industries, Queensland, Australia, 2004)

**Table 2.2:** Comparision of taro nutritive values with other non-graminaceous tropical crops

| Crop | Food Energy (KJ) | Moisture (%) | Protein (g) | Fat (g) | Protein /energy ratio (g/1000Kcal) | Fibre (g) | Total CHO & Fibre (g) | Ash (g) | Ca (mg) | P (mg) | Fe (mg) | K (mg) | Na (mg) | Carotene (ug) | Thiamin equi. (mg) | Riboflavin (mg) | Niacin (mg) | Ascorbic acid (mg) | Folic acid (ug) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cassava | 565 | 65.5 | 1 | 0 | 7 | 1 | 32 | 1 | 26 | 32 | 0.9 | 394 | 2 | 0 | 0.05 | 0.04 | 0.6 | 34 | 24 |
| Sweet potato | | | | | | | | | | | | | | | | | | | |
| White | 452 | 72.3 | 1 | 0 | 15 | 0.8 | 25 | 1 | 21 | 50 | 0.9 | 210 | 31 | 35 | 0.14 | 0.05 | 0.7 | 21 | 52 |
| Yellow | 481 | 70 | 1.2 | 0 | - | 0.8 | 27 | 1 | 36 | 56 | 0.9 | 30 | 36 | 1680 | 0.12 | 0.05 | 0.6 | 30 | - |
| Potato | 335 | 78.3 | 2 | 0 | 27 | 0.4 | 19 | 1 | 9 | 55 | 0.7 | 451 | 7 | 30 | 0.11 | 0.04 | 1.2 | 14 | - |
| Yam | 452 | 71.8 | 2 | 0 | 21 | 0.5 | 25 | 1 | 22 | 39 | 1 | 294 | 10 | 0 | 0.1 | 0.04 | 0.07 | - | - |
| Taro and Tannia | 393 | 75.4 | 2.2 | 0 | - | 0.8 | 21 | 1 | 34 | 62 | 1.2 | 448 | 10 | tr | 0.12 | 0.04 | 1 | 8 | - |
| Giant taro | 255 | 83 | 0.6 | - | - | - | 15 | - | 30 | 50 | 1 | - | - | 0 | 0.05 | - | - | 5 | - |
| Giant swamp taro | 548 | - | 0.9 | - | - | - | 31 | - | 334 | 56 | 1.2 | - | - | 0 | 0.05 | 0.07 | 0.88 | - | - |
| Elephant yam | 339 | 78.5 | 2 | - | - | - | 18 | - | 38 | 38 | 2.4 | 418 | - | 0 | 0.06 | 0.12 | 1.7 | 8 | - |
| Taro leaves | 255 | 81.4 | 4 | - | - | - | 12 | - | 162 | 69 | 1 | 963 | - | 5535 | 0.13 | 0.34 | 1.5 | 63 | 163 |
| Sweet potato tips | - | 86.4 | 2.7 | - | - | - | - | - | 74 | - | 4 | - | - | 5580 | 0.32 | - | - | 41 | - |
| Banana | 425 | 71.6 | 1.2 | 0 | - | 0.6 | 26 | 1 | 12 | 32 | 0.8 | 401 | 4 | 225 | 0.03 | 0.04 | 0.6 | 14 | - |
| Plantain | 476 | 68.2 | 0.9 | 0 | - | 0.4 | 30 | 1 | 19 | 38 | 0.6 | - | - | 475 | 0.15 | 0.06 | 0.7 | 11 | - |

(Oke, 1990)

### 2.1.4 Economical importance and uses of taro

In South Pacific Island countries and parts of Africa, taro is a staple food crop (Lebot and Aradhya, 1991; Opara, 2001). About 400 million people include taro in their diets; its highest percentage contribution to the diet occurs in the Pacific Islands (Ivancic, 1992). In Tonga, for example, tubers represent almost half the nations calorie intake of which about 40 % is taro. Similarly, in Solomon Islands, about 10 % of people's dietary calories is derived from taro and 30 % from other tubers. Moreover, in Samoa, prior to a devastating outbreak of taro leaf blight disease (TLB), virtually all the populations' dietary intake from tubers (one-fifth of the overall diet) came from taro (CTA, 2003). Taro is one of the few major staple foods where both the leaf and underground parts are equally important in the human diet (Lee, 1999). As such, it has attained considerable economic importance as a fresh crop in many large islands in the region such as Samoa, Fiji and others (Hanson and Imamuddin, 1983). Due to its increasing demand in the local and overseas market, taro production has increased tremendously in the Oceania region over the years (Table 2.3). It is now becoming one of the major export commodities, and is bringing in substantial foreign exchange to some Pacific Island countries. For example, Fijian taro exports have been steadily increasing since 2001 (Table 2.4).

Large quantities of taro are produced in the Asia/Pacific region, with the corm being boiled, baked or fried and consumed with fish, coconut preparations, etc. A favorite and peculiarly Pacific way to prepare taro is to roast it on hot stones in dug-out earth ovens. This is quite common when taro is used in feasts and ceremonies. Young taro leaves are a major vegetable throughout Melanesia and Polynesia where they are usually boiled, cooked in coconut cream or wrapped in banana or breadfruit leaves and cooked on hot stones. The processed and storable form of taro is the taro chip and Poi. Taro chips are prepared by peeling the corm, washing, slicing into thin pieces and blanching; the pieces are then fried in oil, allowed to cool, then drained and packed.

**Table 2.3:** Taro production in the Oceania Region, 1994-2004.

| Year | 2004 | 2003 | 2002 | 2001 | 2000 | 1999 | 1998 | 1997 | 1996 | 1995 | 1994 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Area Harvested (Ha) | 50130 | 49020 | 49000 | 46300 | 45372 | 43564 | 42696 | 39971 | 37330 | 36586 | 39184 |
| Yield (Kg/Ha) | 72871 | 72868 | 72878 | 71896 | 69025 | 69862 | 66495 | 66436 | 65983 | 68886 | 65386 |
| Production (Mt) | 365300 | 357200 | 357100 | 332880 | 313181 | 304347 | 283905 | 265550 | 246313 | 252026 | 256210 |

(CAB International, 2005)



**Table 2.4:** Taro exports from Fiji Islands, 2000-2005.

| Year | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 |
|---|---|---|---|---|---|---|
| Quantity (Kg) | 7,381,360 | 6,020,609 | 6,617,689 | 9,007,650 | 9,637,923 | 9,959,468 |
| Value ($FJ) | $12,329,265 | $10,274,669 | $11,055,890 | $14,943,504 | $18,690,004 | $19,006,178 |

(Fiji Islands Bureau of Statistics, 2006)

Poi is a sour paste made from boiled taro and its production and utilization is quite limited – mainly in the Hawaiian Islands.

Griffin (1982) has emphasized other important economic uses of taro. For example, the development of taro silage and its use as animal feed especially for swine, the potential of taro alcohol as a fuel for remote islands and the potential of taro starch as a raw material in cosmetic and plastic manufacture. Furthermore, taro flour and other products have been used extensively for infant formulae in the United States and have formed an important constituent of proprietary canned baby foods (Lee, 1999).

### 2.1.5 Cultural importance of taro

Taro has evolved with the cultures of the people of the Asia and Pacific region and as a result, it has acquired considerable socio-cultural importance. It is considered a prestige crop and the crop of choice for royalty, gift-giving, traditional feasting and the fulfillment of social obligations. It features prominently in the folklore and oral traditions of many cultures in Oceania and South-east Asia. Samoa and Tonga each have prominent depictions of taro on their currencies (Onwueme, 1999). Moreover, in Hawaii, images of taro and taro farmers can be found throughout the islands, in murals, posters, original arts and other visuals, where its symbolic importance reflects its continuing role as a common food and common element in the agricultural landscape (Matthews, 1998). The socio-cultural attachment to taro has meant that taro itself has become a symbol of cultural identification, such that the people of Pacific Island origin continue to consume taro wherever they may live in the world. This is one of the means of maintaining links with their culture; consequently, this cultural attachment to taro has spawned a lucrative taro export market to ethnic Pacific Islanders living in Australia, New Zealand and western North America (Onwueme, 1999). Taro is also used as a traditional medicine with root extracts used to treat rheumatism and acne, while leaf extracts are used for clotting blood, neutralizing snake poison and as a purgative medicine (Thinh, 1997).

### 2.1.6 Sexual reproduction and propagation

Taro is mainly vegetatively propagated (Shaw, 1975; Strauss *et al.*, 1979; Kuruvilla and Singh, 1981; Ivancic, 1992), but the complete life cycle (Figure 2.3) consists of both sporophyte and gametophyte generations (Ivancic, 1992). The gametophytic generation is limited to egg and pollen.

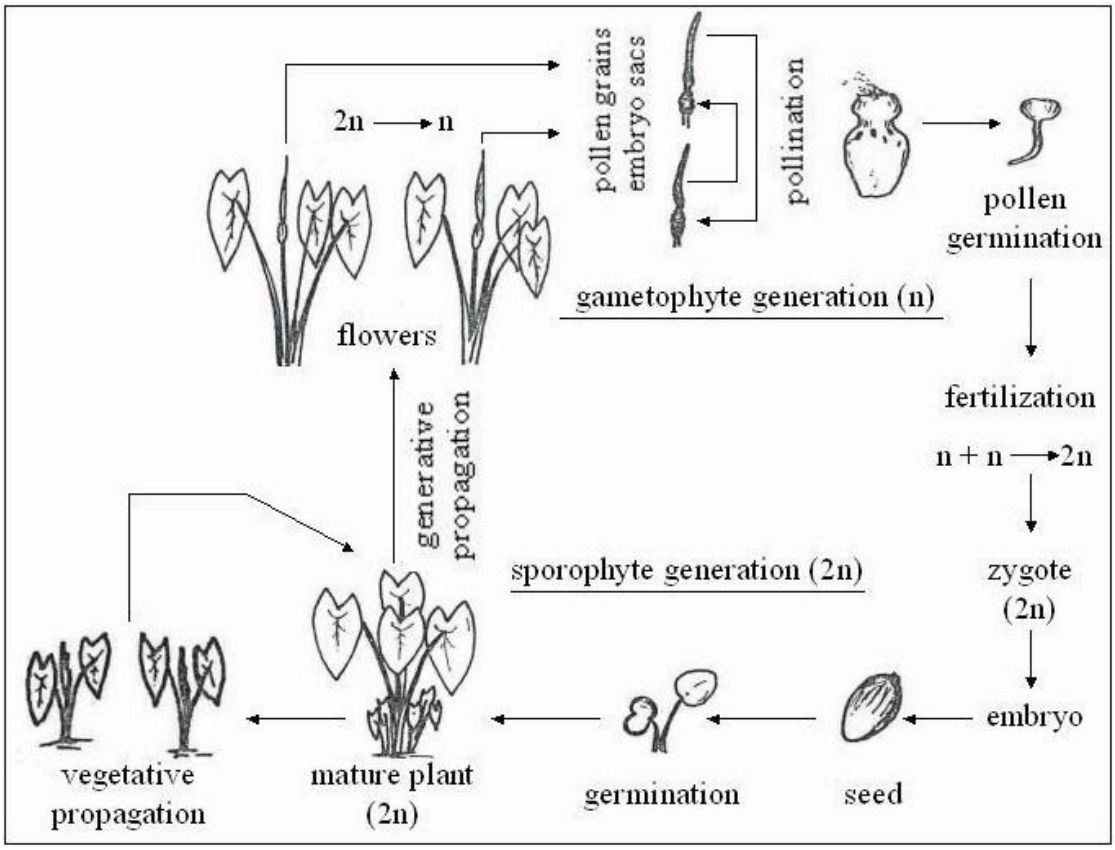


**Figure 2.3:** The life cycle of taro (*Colocasia esculenta*) (Ivancic, 1992)

Due to vegetative/clonal propagation, there is almost no genetic variation within the cultivars although somatic mutations do occur. Consequently, the introduction of new cultivars in response to changes in pest and disease populations or changes in climatic conditions takes much longer (Ivancic, 1992).

Sexual hybridization of taro is now well documented and techniques for pollinating and growing seedlings have been established (Shaw, 1975; Hanson and Imamuddin, 1983; Wilson, 1990; Ivancic, 1992; Singh *et al.*, 2001; Tyagi *et al.*, 2004). Sexual hybridization is one way to generate new cultivars with improved qualities (Strauss *et al.*, 1979). Extensive breeding programs have been carried out in Samoa, Papua New Guinea and Hawaii to produce cultivars with resistance to Taro Leaf Blight (TLB) along with high yields (Lebot and Aradhya, 1991; TaroGen, 1999; Singh *et al.*, 2001). From these programs, promising new cultivars resistant to TLB have been produced in Hawaii (Trujillo *et al.*, 2002), PNG and Samoa (SPC, 2002a).

Even though sexual hybridization of taro is showing some promising results, it is a labor intensive and lengthy process in terms of field preparation, planting of parents, induction of flowering, pollination, development and maturation of fruit heads and seed harvesting. In addition, the germination and planting of seedlings and screening processes take several years. “It often takes 10 years or more from the time you make a pollination, until the new, improved cultivar finally reaches a large number of farmers” (Wilson, 1990). Further, viable seed production depends on compatibility of parents as well as the vagaries of weather, pests and diseases.

One technique used for intensive clonal propagation of taro is tissue culture (Jackson, 1977), which involves excising taro apical and axillary buds, decontaminating them and culturing *in vitro* in sterile nutrient medium. The cultured bud can then be grown into a plantlet, and intensive sucker production induced by the application of plant growth regulators. If the meristem is cultured rather than the whole bud, it is possible to eliminate viruses, which are particularly problematic in vegetatively propagated crops.

### 2.1.7 Diseases and pests of taro

In many countries taro is being replaced by sweet potatoes and cassava due to pests and disease problems, which are becoming a limiting factor for taro production (Ivancic, 1992). Many taro pests and disease-causing pathogens have been reported.

Viruses are one of the most important pathogens with some infections resulting in severe yield reductions and plant death. Currently five viruses: Dasheen mosaic virus (DsMV), Taro bacilliform virus (TaBV), Colocasia bobone disease virus (CBDV), Taro vein chlorosis virus (TaVCV) and Taro reovirus (TaRV) have been reported to infect taro with varying distribution throughout the Pacific Islands. The main effect of virus infection is a reduction in corm size and quality, with yield losses of up to 20 % being reported (Revill *et al.*, 2005). The presence of taro viruses currently restricts the international movement of taro germplasm. This has serious implications since many countries are denied access to agronomically elite lines including selected traditional cultivars.

Fungi are also important taro pathogens of which *Phytophthora colocasiae* is most significant as it causes Taro Leaf Blight (TLB). TLB has been present in Papua New Guinea, Federated States of Micronesia, Northern Mariana Islands, Palau and the Solomon Islands for over 50 years. An outbreak in the Solomon Islands after World War II resulted in a permanent shift in some parts of the country away from taro to sweet potato and cassava production (Coleman *et al.*, 2003). TLB disease struck American Samoa and Western Samoa in 1993-1994. Since all Samoan cultivars were susceptible to this fungus production was devastated. Disease resistant cultivars were not introduced until early 1997, after export from Samoa had dramatically reduced causing losses of many millions of dollars (USDA, 2001). In 2002, the blight epidemic reduced production sufficiently in the Morobe Province in PNG such that food aid was requested. A number of the Pacific Island countries, namely Fiji, Tonga, Cook Islands and Vanuatu are considered susceptible to an outbreak as they share the common blight-enhancing conditions of rainfall greater than 2,500 mm annually that is spread relatively evenly throughout the year (Coleman *et al.*, 2003). This shows that

TLB constitutes a significant threat to food security and economy in those Pacific Island countries which do not have resistant varieties and where taro is a major staple and an export commodity.

Amongst the pests, taro beetle belonging to the genus *Papuana* is of great concern. The adult beetles fly from their breeding sites to the taro field and tunnel into the soil just at the base of the taro corm. They then proceed to feed on the growing corm leaving large holes that reduce the eventual market quality. Further, the wounds they create while feeding promote the attack of rot-causing organisms. The feeding activity can cause wilting and even death of the affected plant (SPC, 2002b).

In summary, diseases and pests, especially TLB and taro beetle are becoming a threat to taro industry in the Pacific. Although improvements in the major grain crops have increased world food production dramatically during the last twenty years, these advances have not brought significant benefits to areas where root crops are major staples (Lee, 1999). This is largely because these crops generate little interest in the research world, and as a rule do not attract donor funds. Much of the research that is carried out on crops these days is funded through the International Agricultural Research Centres (IARCs). Unfortunately, no IARC has the mandate to conserve and carry out research on taro. However, these staple root crops are very important to food security in many Pacific Island countries, and any research benefiting their production would be worthwhile and beneficial to the entire Pacific region.

One way in which taro could be improved is through utilizing biotechnologies such as somatic embryogenesis and recombinant DNA technologies to accomplish transfer of desirable traits, hence producing improved cultivars of taro. Developing somatic embryogenesis and transformation systems for taro would boost the production of good quality taro for all Pacific Island countries where taro has been a major subsistence and export commodity.

## *2.2 Somatic Embryogenesis and Its Application*

### 2.2.1 What is somatic embryogenesis?

In nature, embryos result from normal fertilization processes by the union of gametes. In plants, however, embryo-like structures can be generated from cells other than gametes (i.e. somatic cells), hence the term somatic embryos (Parrott, 2000). As somatic embryos are not the result of a fertilization event they are genetically identical to the parent tissue and are therefore clones.

Somatic embryogenesis may be direct or indirect. Indirect somatic embryogenesis involves dedifferentiation of organized tissue into callus prior to embryo production whereas direct somatic embryogenesis involves production of embryo from organized tissue without an intervening callus phase (Slater *et al.*, 2003).

Irrespective of the mode of production, it has been argued that the anatomical and physiological features of somatic embryos are highly comparable to zygotic embryos (Cheng and Raghavan, 1985; Boxus, 1999; Gray, 1992; Zimmerman, 1993; Bandyopadhyay and Hamill, 2000; Parrott, 2000). This claim is supported by the work of Bandyopdhyay and Hamill (2000) on *Eucalyptus nitens,* which revealed that both somatic and zygotic embryos have strong similarities in terms of their overall size, morphology and internal cellular organization.

Zimmerman (1993) further argued that the morphological and temporal development of somatic embryos are very similar to that of zygotic embryos and that they both proceed through a series of distinct stages (Figure 2.4), namely globular, heart, torpedo and cotyledon or plantlet stages for dicotyledons (Zimmerman *et al.*, 1989; Zimmerman, 1993; Mandal and Gupta, 2002) and globular, elongated, scutellar and coleoptilar stages for monocotyledons (Gupta and Conger, 1999; Godbole *et al.*, 2002). These stages typically span a period of several days.

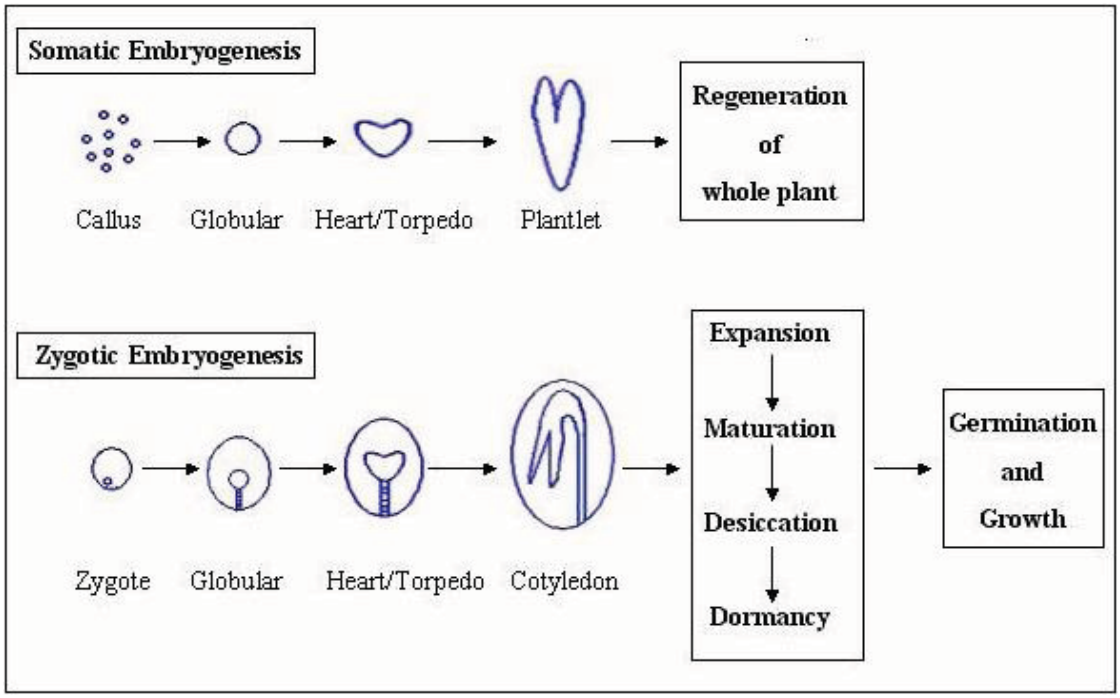


**Figure 2.4:** Embryogenesis in dicotyledonous plants: somatic and zygotic (Zimmerman, 1993)

The model plant for studying dicotyledonous somatic embryogenesis has been carrot (Drew, 1972; Jones, 1974; McWilliam *et al.*, 1974; Smith and Krikorian, 1989; Zimmerman *et al.*, 1989; Smith and Krikorian, 1990; Jong *et al.*, 1992; Zimmerman, 1993). Initially, small globular embryos form which then undergo isodiametric growth and establish bilateral symmetry. These then develop into the heart stage embryo in which both cotyledons and root and shoot meristems are clearly established. The development proceeds with the formation of torpedo and subsequently plantlet stages. The plantlets contain green cotyledons, elongated hypocotyls and developed radicals with very fine root hairs (Zimmerman, 1993).

In monocotyledonous embryogenesis, especially in graminaceous species, the transition from globular stage follows a series of events all occurring simultaneously. This includes the development of scutellum, initiation of the coleoptilar notch, and tissue differentiation with the development of embryogenic vascular system and

accumulation of intracellular storage substances. At the final stages of maturation, the coleoptile undergoes enlargement and the embryo axis becomes more developed. The embryo axis develops laterally and parallel to the scutellum, while the root apical meristem is embedded and the shoot apical meristem develops externally and is protected by the coleoptile (Gray, 1996). The first leaf primordium appears at the base of the shoot apex while development of leaf primordia is preceded by the formation of root(s) from the root meristem. As the plantlet develops, numerous root hairs develop on the main root (Meinke, 1991).

### 2.2.2 How is somatic embryogenesis used?

Somatic embryogenesis is a valuable tool in plant biotechnology and can be utilized in a number of ways (Zimmerman, 1993; Bandyopadhyay and Hamill, 2000; Saiprasad, 2001):

- For large-scale clonal propagation of elite cultivars it provides an alternative approach to conventional micropropagation.
- Synthetic (artificial) seed can be developed from somatic embryos potentially facilitating broad-acre direct seeding of elite cultivars or providing a means of moving germplasm in a less fragile form than *in vitro* plantlets.
- Embryogenesis via callus or secondary embryogenesis may assist in the application of gene transfer techniques for further genetic improvements.
- Somatic embryogenesis systems offer potential models for studying molecular, regulatory and morphogenetic events in plant embryogenesis.

### 2.2.3 The advantages of somatic embryogenesis for mass propagation

Large-scale production of plants through the multiplication of embryogenic cell lines is the most commercially attractive application of somatic embryogenesis (Jiménez, 2001), and is the most practical application of this technique to benefit agriculture. It has many advantages over conventional micropropagation in this respect:

- It permits the culture of large numbers of somatic embryos. For example, 60,000 to 1.35 million somatic embryos can be regenerated per litre of medium.

- During regeneration, root and shoot formation is simultaneous thus eliminating the need for a root induction phase as with conventional micropropagation.
- The mode of culture permits easy scale-up and subculture with low labour inputs.
- Cultures can be manipulated such that embryo formation and germination can be synchronized maximizing plant output while minimizing labour inputs.
- As with zygotic embryos, somatic embryos dormancy can be induced, hence long-term storage is possible.

### 2.2.4 Limitations of somatic embryogenesis

Even though somatic embryogenesis offers great potential, it also has some limitations. Firstly, the development of somatic embryos tends to be non synchronous (Zimmerman, 1993; Zegzouti *et al.*, 2001) thus embryos of all stages can be present in one culture system. However, Fujimura and Komamine (1979) demonstrated that the development of carrot somatic embryos can be synchronized, for instance, by grouping cell aggregates of similar size and density from suspension cultures using sieving and density gradient centrifugation. Even though synchronization of somatic embryos can be achieved using sieving and density gradient centrifugation, it appears that the percentage of somatic embryos regenerated is affected by the size of cell aggregates. Chee and Cantliff (1989) pointed out that a decrease in the size of cell aggregates led to a reduction in the percentage of somatic embryo formation in sweet potato. In spite of this phenomenon, selection of only the highly regenerable portion of a cell culture would still be a more efficient propagation system than conventional micropropagation. For example, the rate of somatic embryo formation in banana suspension cultures can be over 100,000 per mL of cells (Cote *et al.*, 1996). Thus, selection of a subgroup of cells and discarding the remainder would still provide many plants.

The second limitation is the stability of cell lines. Over a period of time, the proportion of cells that enter or complete embryogenesis decreases so that eventually, regeneration may become impossible. This in fact could be an advantage as prolonged

time in culture can lead to the accumulation of mutations (somaclonal variations), which can cause morphological abnormalities such as pluricotyledony, multiplex apex formation and fused cotyledons (Evans *et al.*, 1983). Thus, being forced to initiate new cultures as old ones lose regenerability may reduce the frequency of somaclonal advantage. The two may in fact be linked with increasing mutations associated with an inability to regenerate. Working with suspension cultures of *Daucus carota*, Evans *et al* (1983) showed that frequently initiating new cultures and maintaining the cultures for less than one year resulted in the regeneration of phenotypically normal somatic embryos and plants. Somaclonal variation also occurs with conventional micropropagation hence new cultures are also initiated on a regular basis. It should be noted, however, that somaclonal variation could have tremendous potential for producing novel and useful varieties.

### 2.2.5 Factors Affecting Somatic Embryogenesis

#### 2.2.5.1 Role of plant growth regulators (PGRs) in the development of somatic embryos

Somatic embryogenesis has been achieved in many agronomical and horticultural significant species such as cassava (Taylor, 1980; Raemakers *et al.*, 1993; Woodward and Puonti, 2001), wheat (Ozias-Akins and Vasil, 1983), maize (Vasil *et al.*, 1984), onion (van der Valk *et al.*, 1992), rice (Suprasanna *et al.*, 1994; Gairi and Rashid, 2004), sweet potato (Liu *et al.*, 1998), mangoes (Ara *et al.*, 1999), peanuts (Parrott, 1999, 2000; Radhakrishnan *et al.*, 2001; Rani and Padmaja, 2005), soybean (Parrott, 1999, 2000; Kim, Park *et al.*, 2004), papaya (Fernando *et al.*, 2001), cotton (Sakhanokho *et al.*, 2001), coffee (Barry-Etienne *et al.*, 2002), banana (Garcia *et al.*, 2002; Strosse *et al.*, 2003), sorghum (Mishra and Khurana, 2003) and sugar cane (Desai *et al.*, 2004; Gandonou *et al.*, 2005).

The most commonly used protocol for induction of embryogenesis involves the induction of callus in an auxin-supplemented medium and somatic embryogenesis upon transfer of callus to a medium low in growth regulators (Cheng and Raghavan, 1985; Smith and Krikorian, 1989; Smith and Krikorian, 1990; Gray, 1992; Zimmerman, 1993). The establishment and maintenance of embryogenic cultures of

nearly all species has relied primarily on the manipulation of growth regulators (Smith and Krikorian, 1990). In particular, the presence of auxin promotes callus proliferation and inhibits differentiation while the removal or decrease in auxin allows somatic embryo development to progress. Morphogenetic changes can be observed upon transferring callus to an auxin-free medium (Cheng and Raghavan, 1985; Zimmerman, 1993). It appears that the removal of auxin from the medium provides the signal for the callus cells to embark on an organized pattern of growth. The fact that embryogenesis can occur upon withdrawal of growth regulators suggests that in the presence of auxin, the proembryonic masses (PEMs) within the culture system may already be "primed" to complete the globular stage of embryogenesis and that the PEMs may also contain products inhibitory to the progress of the embryogenesis program (Zimmerman, 1993). Consequently, the removal of auxin may result in the inactivation of genes responsible for the presence of these inhibitory products, enabling the embryogenesis program to proceed. The observation that some carrot cell lines were only able to develop to the globular stage in the continued presence of auxin also suggests that new gene products are needed for the transition to the heart stage and these new products are synthesized only when exogenous auxin is removed from the culture medium (Zimmerman, 1993).

Auxins are also known to be the principal agents responsible for the establishment of cell polarity (apical-basal axis). It has been suggested that the polar transport of auxin in early globular embryos is essential for the establishment of bilateral symmetry during plant embryogenesis (Liu *et al.*, 1993). For the induction of the process leading to polarity, relatively high levels of endogenous, free indole-3-acetic acid (IAA) may be necessary. However, once induction has occurred, those high levels of IAA must be reduced to allow the establishment of the auxin gradient. If the levels are too low or high or do not diminish after the induction, the gradient cannot be formed and thus somatic embryogenesis cannot progress (Jiménez, 2001). Failure in the establishment of proper gradient using inhibitors of cell IAA efflux carrier proteins such as naphthylphthalamic acid (NPA) caused the formation of abnormal embryos in Indian mustard (*Brassica juncea*) (Liu *et al.*, 1993) and *Fucus distichus* (Basu *et al.*, 2002). This suggests that auxin plays a role in the formation of apical-basal pattern in

embryo development and that a proper distribution of auxin (a gradient) is required for the establishment of polarity, which is a critical event in plant embryogenesis (Sun *et al.*, 2004).

Although auxins are known to be the principal agents responsible for cell polarity, other stimuli have an effect and hence influence the efficiency of somatic embryogenesis. For example, in white clover (*Trifolium repens* L.), cytokinin promoted the formation of embryogenic cells from the epidermis of immature zygotic embryos (Dodeman *et al.*, 1997), thus it was proposed that exogenous growth regulators modify cell polarity by interfering with pH gradients or electropotential at a cellular level. Consequently, it could be argued that the combined effect of multiple growth regulators and other components of the medium might influence both the establishment of cell polarity and the subsequent cellular processes leading to the formation and development of normal somatic embryos. That somatic embryogenesis commonly occurs using exogenous applications of auxin and its withdrawal, does not mean that this hormone alone is responsible as the plant tissue itself may synthesize endogenous auxins and other hormones.

Cytokinins are known to stimulate cell division and, as such, they are also suitable candidates for induction of somatic embryogenesis and caulogenesis. For example, in some cases thidiazuron (TDZ) has stimulated *in vitro* shoot regeneration and somatic embryogenesis (Thinh, 1997; Mithila *et al.*, 2003; Srangsam and Kanchanapoom, 2003; Lin *et al.*, 2004; Verma *et al.*, 2004). Like many synthetic plant growth regulators, TDZ was originally developed as a herbicide, in this case a cotton defoliant, with cytokinin-like qualities (Panaia *et al.*, 2004). It has been suggested that TDZ is more effective than other cytokinins used for somatic embryogenesis (Thinh, 1997; Lin *et al.*, 2004). The effects of TDZ occur at lower concentrations than other cytokinins and it has been suggested that it either directly promotes growth due to its own biological activity or through inducing the synthesis and/or accumulation of endogenous cytokinins or auxins. The latter could explain the effectiveness of TDZ as it may be mediating levels of endogenous auxin/cytokinin levels within the cultured tissue (Visser *et al.*, 1992; Panaia *et al.*, 2004).

The above details support the argument of Vasil and Vasil (1981) that "the initiation of shoots and embryos cannot be ascribed to any one plant growth regulator", although the most successful procedure appears to be the transfer of tissues from a medium containing auxin (commonly 2,4-D) to a medium devoid of this synthetic auxin or containing a very low concentration. It also appears that different species have different levels of sensitivity towards various plant growth regulators; hence, their response to embryogenesis is variable. For instance, in experiments with lucerne (*Medicago sativa L.*), 2,4-D either separately or in combination with kinetin or naphthalene acetic acid (NAA) and benzylaminopurine (BAP) could not stimulate the formation of embryogenic callus. However, this callus could be induced in medium containing IAA and zeatin (Kim, Kim *et al.*, 2004). In addition, as shown by Panaia *et al* (2004), different responses can be obtained from species belonging to the same family, for example, in the Restionaceae family, *Desmocladus flexuosus* responded to BAP and TDZ while *Baloskion tetraphyllum* responded only to 2,4-D. This clearly indicates that the different requirements for plant growth regulators operate at species, and even at cultivar level. Hence, in any research on somatic embryogenesis, a range of plant growth regulators should initially be used, so that the optimal "stimulant" combination can be identified.

#### 2.2.5.2 Explant, plant genotype and culture conditions are crucial for somatic embryogenesis

Variations in *in vitro* response have been known to occur due to a number of different factors, such as basal medium (Zegzouti *et al.*, 2001), explant source (Sharma and Rajam, 1995; Haliloglu, 2002) and genotype (Radhakrishnan *et al.*, 2001; Kim *et al.*, 2003).

Various explants have been utilized to initiate somatic embryogenesis including anthers, pollen, ovaries, (Cheng and Raghavan, 1985; Songstad and Conger, 1986; Jayasree *et al.*, 1999), leaves (Cheng and Raghavan, 1985; Birhman *et al.*, 1994), petioles and stems (Cheng and Raghavan, 1985; Reynolds, 1986), immature and mature embryos (Gray, 1992; Smith and Krikorian, 1989) and mature cotyledons (Venkatachalam *et al.*, 1999; Barry-Etienne *et al.*, 2002).

Even though a variety of explants can be utilized, the correct developmental stage of the explants is also crucial for the initiation of embryogenic callus. Lu and Vasil (1982) demonstrated that when the explant stage in *Panicum maximum* was incorrect, only a soft, friable and translucent callus with no embryogenic potential was produced. In addition, young or juvenile explants produced more somatic embryos than older explants (Woodward and Puonti, 2001; Panaia *et al.*, 2004). As a further complexity, different explants tissues from the same mother plant produced embryogenic callus at different frequencies (Zhang *et al.*, 2001) and required different concentration of growth regulators for the induction of somatic embryos (Sharma and Rajam, 1995). The different endogenous phytohormone levels of various explants tissues might be a factor influencing the requirements of exogenous growth regulators. As a general rule, the type and age of explants has an impact on somatic embryogenesis, and highlights the observation that young, dividing and possibly less differentiated cells are more likely to be stimulated towards the embryogenic pathway than older cells.

The effect of genotype on somatic embryogenic competence has been clearly shown. For example, out of the five cultivars of Hybrid Tea roses (*Rosa hybrida* L) investigated, somatic embryogenesis could only be induced in two (Kim *et al.*, 2003). A similar phenomenon has been observed in red clover (McLean and Nowak, 1998), peanut (*Arachis hypogea*) (Radhakrishnan *et al.*, 2001) and Chinese cotton (Zhang *et al.*, 2001). The presence of varying levels of endogenous phytohormones, particularly cytokinins, in different genotypes might influence their response to somatic embryogenesis. Wenck *et al* (1988) observed that genotypes of orchard grass in which embryogenesis was difficult to induce contained considerably higher levels of endogenous cytokinins than embryogenic genotypes.

The recalcitrance of some species can be overcome by manipulating other media components (Birhman *et al.*, 1994). This has been substantiated by experiments of Panaia *et al* (2004) whereby somatic embryogenesis in *Baloskion tetraphyllum* was achieved using half-strength MS salts with 0.22 mg/L 2,4-D, with approximately 14,000 somatic embryos were obtained from 1 g of plant material. In another study,

Samson *et al* (2006) demonstrated that a two or four-fold dilution of the MS salts increased the development rate of *Coffea* embryogenic callus by 2.6 and 5.7, respectively, in comparison to full-strength MS salts. Conversely, Groll *et al* (2002) showed that media with altered macro and micro nutrient salt concentrations affected the development and germination capability of cassava somatic embryos, with half-strength and full-strength MS medium proving superior for development and germination compared to quarter-strength MS. This clearly demonstrates that medium modifications, in particular manipulating the concentrations of inorganic salts and vitamins, can have a significant effect on somatic embryogenesis possibly through altering the osmotic potential of the medium.

In addition to modifying salts and vitamins, Gairi and Rasheed (2004) showed that by subjecting explants or callus culture to auxin (2,4-D) treatment for several days followed by their transfer to medium containing TDZ, a previously non-responsive or recalcitrant cultivar of rice could be induced to become responsive to somatic embryogenesis. This introduces another variable in the development of methods to induce somatic embryogenesis, that is, brief "pulses" on one medium followed by transfer to another.

The success in initiating embryogenic callus, somatic embryos and the subsequent recovery of viable plants is not readily achievable for many species. Induction may, in fact, demand long and complex treatments or procedures where non-embryogenic cells can be induced to an embryogenic state by a variety of procedures including treatment with plant growth regulators and various other chemicals and manipulations to light, temperature and pH. Therefore, in order to determine the effective conditions for somatic embryogenesis in different species, the required conditions must be determined empirically by manipulating the many factors that contribute to the culture conditions (Jiménez, 2001).

### 2.2.5.3 The effect of light and activated charcoal

Many plants are rich in phenolic compounds. Therefore, after tissue injury, such compounds will be oxidized by polyphenol oxidases and the tissue will become brown. The oxidation products are known to not only darken the tissue, but also to inhibit activity of various proteins which may have an inhibitory effect on somatic embryogenesis (Evans *et al.*, 1983).

Smith and Krikorian (1990) reported that somatic embryogenesis in carrot failed to occur under continuous light unless activated charcoal filter papers were used. Growth under white light had been associated with elevated phenolic production and an increased level of abscisic acid (Evans *et al.*, 1983; Smith and Krikorian, 1990). Activated charcoal removes inhibitors of embryogenesis, in particular phenylacetic acid, benzoic acid derivatives and other colourless toxic compounds by adsorption (Drew, 1972; Srangsam and Kanchanapoom, 2003). Moreover, activated charcoal also has been shown to absorb 5-hydroxymethylfurfural, an inhibitor formed by the degradation of sucrose during autoclaving, as well as substantial amount of auxins and cytokinins. Consequently, apart from absorbing and removing inhibitors that would prevent growth, it may also adsorb and reduce the levels of growth regulators that would otherwise stimulate callus initiation, growth and proliferation. Therefore, it has been suggested that cultures should be maintained in reduced light intensity or in darkness, as this will minimize the production of inhibitory compounds from tissues in the culture medium (Evans *et al.*, 1983). In addition, this will also minimize or negate the inclusion of activated charcoal in the medium, thus ensuring that the potential of the growth regulators present in the medium will not be compromised.

### 2.2.5.4 The effect of other biochemical factors on somatic embryogenesis

Certain bioactive compounds such as the amino acids, glutamine, proline and tryptophan and polyamines such as putrescine have been identified as enhancers of somatic embryogenesis in some species. Their efficacy in embryogenesis has been attributed to their contribution to various cellular processes such as improving cell signaling processes in various signal transduction pathway (Lakshmanan and Taji,

2000), as precursor molecules for certain growth regulators (Siriwardana and Nabors, 1983; Ribnicky *et al.*, 1996; Jiménez and Bangerth, 2001a; Jiménez and Bangerth, 2001b) or regulators of DNA synthesis (Kevers *et al.*, 2000; Astarita *et al.*, 2003).

In plant tissue culture, the use of glutamine is recognized as assisting in the growth of plant tissues. Glutamine is utilized in different metabolic pathways in various parts of the plant at different stages of development (Ogita, 2005). Endogenous and exogenous glutamine played an important role in embryogenic tissues proliferation and development in Japanese red cedar (*Cryptomeria japonica)* (Ogita *et al.*, 2001); a high concentration of glutamine stimulated the production of embryogenic aggregates and maintained the embryogenic capacity of the tissue. In addition, David *et al* (1984) reported that a high concentration of glutamine in the medium stimulated growth of *Pinus pinaster* cell cultures, while low concentrations resulted in a limited capacity for cell growth. Moreover, increased levels of glutamine in the medium has been shown to reduce the lag phase of Douglas-fir cell growth. Cells observed during the exponential phase of glutamine-stimulated growth displayed morphologies typical of rapidly growing cells that were richly cytoplasmic, dense, round and with small vacuoles, whereas cells grown for the same period on medium without glutamine displayed morphologies typical of stationary phase in that they were highly vacuolated and elongated (Kirby, 1982). It has also been reported that glutamine promotes somatic embryo maturation, enhances the synthesis of storage reserves in the developing somatic embryos and improves embryo germination and continued development (Rodríguez *et al.*, 2006).

Glutamine is a rich source of organic nitrogen in a reduced form and is one of the intermediates in the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, the major route for inorganic nitrogen assimilation. The enzyme glutamine synthetase (GS) catalyses the amidation of glutamate to generate glutamine at the expense of ATP hydrolysis and the enzyme glutamate synthase (GOGAT) catalyses the reductive transfer of amide nitrogen to 2-oxoglutarate for the generation of two molecules of glutamate, one of which is recycled for glutamine biosynthesis. Thus, the availability of glutamate plays a central role in the biosynthesis of the major nitrogen compounds

in plants, including other amino acids, nucleic acids, polyamines and chlorophyll (Ogita *et al.*, 2001; Rodríguez *et al.*, 2006). Since both glutamine and glutamate are directly involved in the assimilation of ammonium ions, direct supply of these amino acids should enhance both the utilization of nitrogen provided inorganically as nitrate and ammonium and their conversion into amino acids and other biomolecules (Durzan, 1982), thus enabling the cells to maintain a high growth and embryogenic potential for a longer period of time (Gamborg, 1970). Therefore, without an adequate supply of glutamine/glutamate, embryogenic tissues may deteriorate and lose their embryogenic characteristics (Ogita *et al.*, 2001).

Some researchers have emphasized that the inclusion of complex organic extracts, such as coconut water (CW), taro extract (TE), potato extract (PE), corn extract (CE) and papaya extract (PAE) are essential for somatic embryogenesis in some species (Ichihashi and Islam, 1999; Islam *et al.*, 2003; Rahman *et al.*, 2004). Moreover, it has also been proposed that these organic extracts are either non-mutagenic or less mutagenic in comparison to conventional growth regulators and, as such, their incorporation in the culture media may minimize somaclonal variations (Lam *et al.*, 1991). However, it can also be argued that organic extracts are undefined components and as such it is not possible to: (i) determine which particular constituent of the extract promotes somatic embryogenesis, and (ii) ensure consistency in the actual extract each time it is prepared.

Jimenez (2001) argued that the culture density of cells also affects somatic embryogenesis. In line with this argument, Umehara *et al* (2004) revealed that both the high-cell-density culture and the conditioned medium derived from high-cell-density culture strongly inhibited somatic embryogenesis in Japanese larch (*Larix leptolepsis*), whereas at low cell-density somatic embryogenesis was high, and at very low cell-culture density most embryogenic clusters did not proliferate and only a few somatic embryos formed. Further investigations showed that the negative effect of the conditioned medium could be attributed to the accumulation of inhibitory factors in the medium, since the addition of activated charcoal to conditioned medium resulted

in the formation of numerous somatic embryos. This suggests the existence of a threshold cell density range, which must be met but not exceeded.

### 2.2.6 Somatic Embryo Development

#### 2.2.6.1 Somatic embryo maturation

An embryogenesis system requires the following steps, which occur in succession: initiation of embryogenic callus from vegetative tissues or cells, maintenance and multiplication of embryogenic cell lines, somatic embryo formation and maturation and finally conversion (germination) of somatic embryos into viable plantlets (Zegzouti *et al.*, 2001).

Maturation is regarded as an essential stage of embryogenesis since the frequency of plant recovery is high from mature embryos. Embryo maturation is a culmination of the accumulation of carbohydrates, lipids and protein reserves, embryo dehydration and a reduction in cellular respiration (Trigiano and Gray, 1996). Thus, maturation is a preparatory stage for embryos for effective germination as Etienne *et al* (1993) stated, "maturation is a transitory, frequently indispensable stage between embryo development and embryo germination phases"; consequently, bypassing the maturation phase will result in precocious germination of embryos with a concomitant reduction in viable plantlets.

Water plays a crucial role in embryo maturation, as dehydration is a major trigger of the process (Etienne *et al.*, 1993). Restricting water uptake using osmoticum has been studied (Etienne *et al.*, 1993; Attree *et al.*, 1995; Gutmann *et al.*, 1996) for its ability to support development of plant embryos while at the same time suppressing precocious germination. Permeating osmoticum, such as sucrose, is frequently used to reduce the water potential of the culture medium resulting in water stress thereby promoting embryo development during *in vitro* culture. However, Attree *et al* (1995) argues that during prolonged culture, such osmoticum will be taken up by the plant cells leading to osmotic recovery. In contrast, non-permeating osmoticum, such as polyethylene glycol-4000 (PEG-4000), can continue to restrict water uptake and so

provide a longer-term drought stress during embryo development. In addition, the rate of desiccation also has an impact on the germination and conversion of somatic embryos into plantlets. For example, rapid desiccation of immature somatic embryos of *Hevea* improved their germination capacity, but their continued development into plantlets was low. In contrast, slow desiccation led to enhancement of germination and was more effective in stimulating conversion into plantlets (Etienne *et al.*, 1993). Slow desiccation resulted in substantial accumulation of starch and protein reserves required for continued development of immature embryos in comparison with rapid dehydration. Therefore, desiccation could be used to enhance germination when the embryo approaches physiological maturity (Etienne *et al.*, 1993). Moreover, Attree *et al* (1995) emphasized that the ability to dry somatic embryos reduces large-scale production costs by providing a means of storing somatic embryos that are produced continuously throughout the year. They can then be germinated synchronously to provide plants of uniform age and size for planting later during a suitable growing season.

Gutmann *et al* (1996) observed that exogenously supplied abscisic acid (ABA) was an important component of maturation medium for *Hybrid larch* somatic embryos. In the absence of ABA, maturation resulted in poorly developed somatic embryos often exhibiting abnormal morphology, non-synchronous development and precocious germination. Subsequently, these somatic embryos had the lowest capacity for germination and plantlet development. In contrast, the presence of ABA in the maturation medium promoted the development of higher quality somatic embryos in large quantities. Under appropriate conditions these somatic embryos germinated and developed into plantlets at a high frequency. It can therefore be concluded that for a somatic embryogenesis system to be practically applied, high frequency embryo formation is of little value unless a large proportion of these embryos are capable of developing into normal plants (Venkatachalam *et al.*, 1999).

### 2.2.6.2 Histology

Histological studies have shown that somatic embryos develop from single cells within a proembryogenic mass (PEM) of cells which itself arises from a single cell (McWilliam *et al.*, 1974; Vasil and Vasil, 1981; Lu and Vasil, 1982; Soh and Bhojwani, 1999; Filonova *et al.*, 2000; Onay, 2000; Fernando *et al.*, 2001; Zegzouti *et al.*, 2001). Plants of such origin are eminently suited for mutant selection, transformation and other genetic analysis. In contrast, plants regenerated via caulogenesis (i.e. adventitious shoots) are believed to be multicellular in origin, and as such could be genetically chimeric. Consequently, the chimeric nature of these plants may not be of use in for selection of mutants or transgenics as the new phenotype may be "hidden" in the first instance or lost over time after a new useful phenotype is selected (Vasil and Vasil, 1981; Lu and Vasil, 1982).

## 2.2.7 Somatic embryogenesis in taro (*Colocasia esculenta* var. *esculenta*)

Regeneration in *Colocasia esculenta* has been reported via caulogenesis (Yam *et al.*, 1991), and somatic embryogenesis (Thinh, 1997; Verma *et al.*, 2004). Initiation of highly regenerable callus is the first step towards an efficient regeneration system. Callus initiation protocols have been well established in *Colocasia esculenta* var. *antiquorum* (Jackson *et al.*, 1977). However, these protocols do not appear to be suitable for cultivars belonging to *Colocasia esculenta* var. *esculenta.* Researchers that have worked with *Colocasia esculenta* var. *esculenta* for many years have not reported callus formation as easily achievable (M.Taylor pers.comm.). Jackson *et al* (1977) reported that callus formation in taro appears to be variety-dependant since their attempts to induce callus from various parts of *Colocasia esculenta* var. *esculenta* failed.

In a small number of cases, induction of organogenic callus in *Colocasia esculenta* var. *esculenta* has been reported. Yam *et al* (1990; 1991) reported some success using axillary buds, half-strength MS macronutrients, one tenth-strength MS micronutrients, full- strength MS vitamins, 25 ml/L taro corm extract (TE) and the plant growth regulator 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). This suggests that taro is

sensitive to high salt concentrations and that low concentration salts based on MS medium would appear to favour callus initiation. High salt concentrations could induce some form of stress, possibly desiccation or ammonium toxicity, which might in turn have an inhibitory effect on callus initiation. Secondly, it has been suggested in the same research that TE is an important requirement for callus initiation. However, TE is an undefined component of a culture medium and so it is not possible to achieve consistency in both the combination and concentration of components in each "individual" taro extract. In addition, the active components in the TE responsible for callus initiation have not been identified.

There are two reports of somatic embryogenesis in taro (Thinh, 1997; Verma *et al.*, 2004). Using MS medium Verma *et al* (2004) developed a two-step protocol (initially on medium supplemented with 2.2 mg/L 2,4-D and 0.44 mg/L TDZ followed by a culture phase with 1.1 mg/L TDZ) to regenerate somatic embryos from petiole explants, with a maximum of 25-30 somatic embryos generated per explant. Thinh (1997) induced somatic embryos from the petiole fourth from the apical dome of the plant using MS medium containing 1.0-2.0 mg/L TDZ. Both report direct somatic embryogenesis. Thinh (1997) states that the somatic embryos were obtained from *C. esculenta* var. *antiquorum* whereas Verma *et al* (2004) does not state whether the variety was *esculenta* or *antiquorum*. To date, there has been no report of an efficient protocol for indirect somatic embryogenesis (that is via an intervening callus and subsequent initiation of cell suspension culture for embryogenic callus) including an efficient and effective regeneration protocol for somatic embryos of *C. esculenta* var. *esculenta.*

## *2.3 Transformation*

### 2.3.1 What is transformation?

Transformation is the introduction of exogenous DNA into plant cells, tissues or organs employing direct or indirect means (Alves *et al.*, 1999). Indirect gene transfer involves the introduction of exogenous DNA by a biological vector such as *Agrobacterium*, whereas direct gene transfer involves introduction of exogenous DNA

by physical or chemical processes such as electroporation, polyethylene glycol mediated DNA uptake, microinjection, silicon carbide fibres and microprojectile bombardment (Taylor and Fauquet, 2002).

### 2.3.2 Applications of transformation

Transformation provides a method of DNA transfer between any living organism (plant, animal, insect, bacterial etc). Further, with DNA synthesis technology becoming more sophisticated, the transgene can be an entirely synthetic sequence. Virtually any desirable trait found in nature can, in principle, be transferred to any chosen organism by transformation (i.e genetic modification) hence the term transgenesis (Betsch, 1994). The first transgenic plants were generated in 1983. These plants were initially transformed with marker genes (genetic markers) but, subsequently, genes conferring traits of potential commercial value were used including improved agronomic qualities, easier processing and other alternatives (Rao and Rohini, 2003). Transgenic plants are also commonly used to complete or substitute mutants in basic research, assisting the studies of complex biological situations such as pathogenesis, genome organization, light perception and signal transduction. One of the major objectives of plant transformation has been to solve agricultural problems without environmental damage (Alves *et al.*, 1999).

Transgenic plants can be categorized into three broad areas of input, output and value-added traits. Lowering production costs by improving crop yields; reducing chemical requirements for pest and disease control, and reducing labour requirements are some of the benefits of input traits to growers. Input traits that are commercially available or being tested in crops include resistance to insects, tolerance to broad spectrum herbicides, resistance to diseases caused by viruses, bacteria, fungi and nematodes and tolerance to environmental stresses such as heat, cold, drought and salinity. Food and fibre products with enhanced qualities are some of the benefits consumers may derive from output traits. Output traits that consumers may one day benefit from include nutritionally enhanced foods with altered carbohydrate, starch, protein or lipid characteristics, higher vitamin or anti-oxidants content, improved taste, increased shelf-life and better ripening characteristics. Value-added traits are obtained by

placing genes into plants that completely change the way they are used. For instance, plants might be used as "manufacturing facilities" to produce large quantities of materials including therapeutic proteins and vaccines, textile fibres, oils for industrial use, detergents and lubricants (Vines, 2001; Becker and Cowan, 2006), hence this category of transgenic plants are called "pharma crops" (Bauer, 2006). Transgenic plants with herbicide tolerance, insect, fungi and virus resistance or modified fruits or flower characteristics and pharma crops have been field evaluated in many countries (Figure 2.5, Table 2.5) (Betsch, 1994; Alves *et al.*, 1999; Rao and Rohini, 2003).

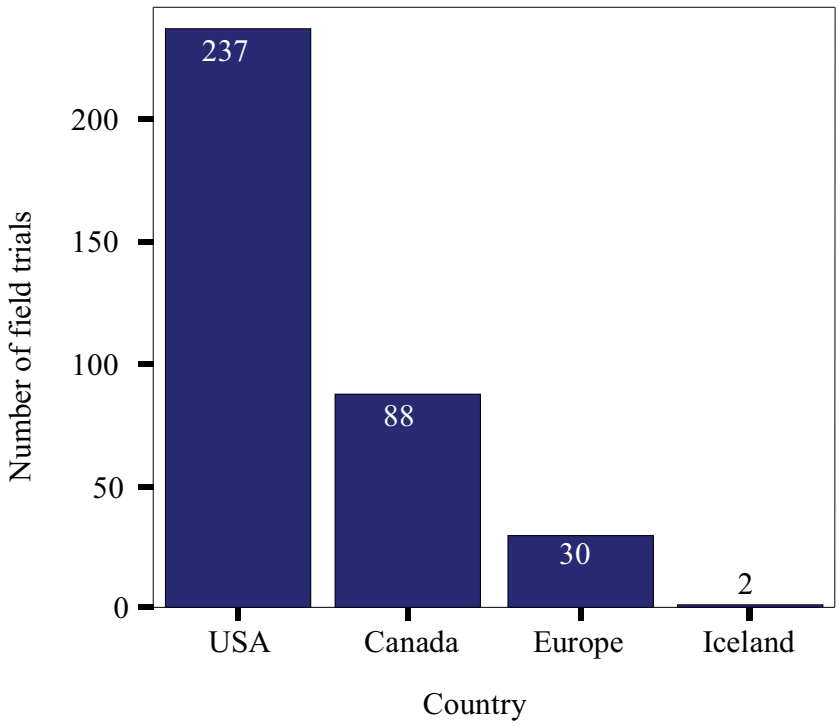


**Figure 2.5:** Number of field trials of pharma crops in various countries, 1991-2005. (Bauer, 2006)

**Table 2.5:** Transgenic plant field trials in various countries, 1992-2007.

| Country | Number of transgenic plants of different species field tested |
|---|---|
| France | 573 |
| Spain | 344 |
| Italy | 295 |
| United Kingdom | 232 |
| Netherlands | 159 |
| Germany | 159 |
| Belgium | 130 |
| Sweden | 87 |
| Denmark | 42 |
| Finland | 22 |
| Portugal | 21 |
| Greece | 19 |
| Hungary | 17 |
| Romania | 14 |
| Ireland | 6 |
| Poland | 5 |
| Czech Republic | 5 |
| Austria | 3 |
| Latvia | 2 |

(GMO Compass, 2008)

The number of countries involved, and the land area utilized, in growing of transgenic crops is increasing dramatically. For example, in 2005, 21 countries including 11 developing and 10 industrialized countries (Table 2.6) grew transgenic crops on an estimated 222 million acres or 90 million hectares. The increase was 9 million hectares, an annual growth rate of 11 % (Figure 2.6). The estimated global net economic benefits to transgenic crop farmers in 2004 were $27 billion ($15 billion for developing countries and $12 billion for industrial countries) (James, 2005).

The major transgenic crops are comprised of maize, oil seed rape, potato, tomato, soybean, cotton, tobacco, barley, wheat and rice, and the most commonly used transgenic traits are herbicide tolerance (35 %), product quality (20 %), insect resistance (18 %), virus resistance (11 %), fungal resistance (3 %), nematode and bacterial resistance and marker or reporter genes (13 %) (Alves *et al.*, 1999; Vines, 2001; Koichi *et al.*, 2002).

In addition to the economic benefits, transgenic crops may also have a positive impact on the environment, since the use of chemicals for pest and disease controls are minimized. For example, the accumulative reduction in pesticides for the period 1996 to 2004 was estimated at 172,500 MT of active ingredient, which is equivalent to a 14 % reduction in the associated environmental impact of pesticide use on these crops, as measured by the Environmental Impact Quotient (EIQ) – a composite measure based on the various factors contributing to the net environmental impact of an individual active ingredient (James, 2005).

**Table 2.6:** Countries cultivating transgenic crops in 2005 (million hectares).

| Rank | Country | Area (million hectares) | Biotech Crops |
|---|---|---|---|
| 1 | USA | 49.8 | Soybean, Maize, Cotton, Canola, Squash, Papaya |
| 2 | Argentina | 17.1 | Soybean, Maize, Cotton |
| 3 | Brazil | 9.4 | Soybean |
| 4 | Canada | 5.8 | Canola, Maize, Soybean |
| 5 | China | 3.3 | Cotton |
| 6 | Paraguay | 1.8 | Soybean |
| 7 | India | 1.3 | Cotton |
| 8 | South Africa | 0.5 | Maize, Soybean, Cotton |
| 9 | Uruguay | 0.3 | Soybean, Maize |
| 10 | Australia | 0.3 | Cotton |
| 11 | Mexico | 0.1 | Cotton, Soybean |
| 12 | Romania | 0.1 | Soybean |
| 13 | Philippines | 0.1 | Maize |
| 14 | Spain | 0.1 | Maize |
| 15 | Colombia | <0.1 | Cotton |
| 16 | Iran | <0.1 | Rice |
| 17 | Honduras | <0.1 | Maize |
| 18 | Portugal | <0.1 | Maize |
| 19 | Germany | <0.1 | Maize |
| 20 | France | <0.1 | Maize |
| 21 | Czech Republic | <0.1 | Maize |

(James, 2005)

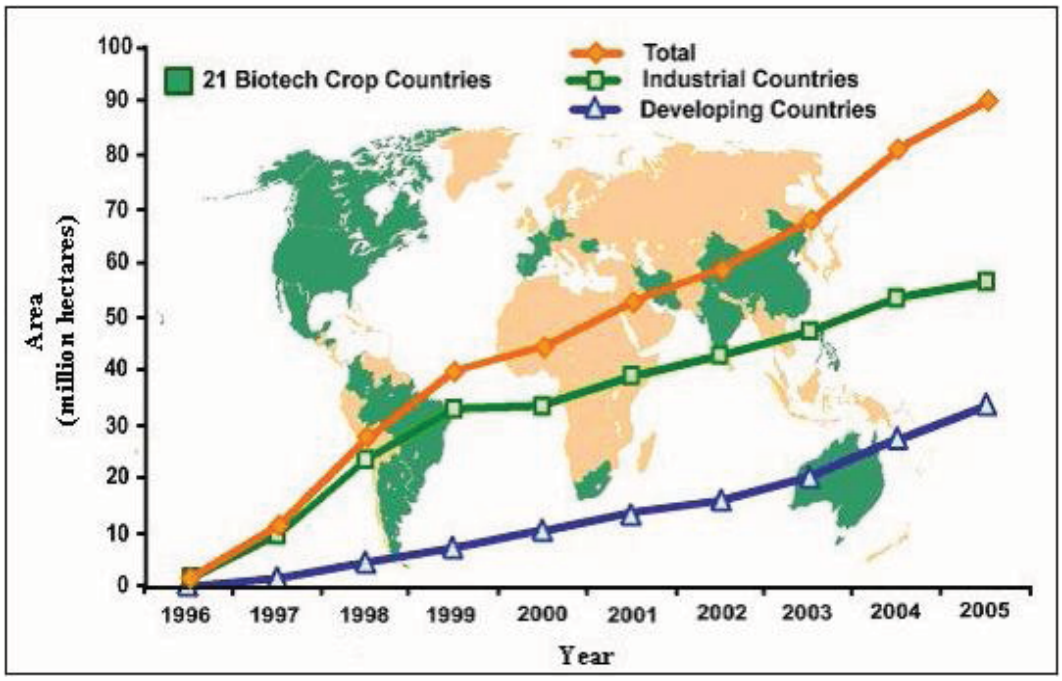


**Figure 2.6:** Global areas of transgenic crops (million hectares) from 1996-2005. (James, 2005)

### 2.3.3 Methods of Plant Transformation

Currently, the two major methods of transformation are *Agrobacterium*-mediated and microprojectile particle bombardment (biolistics) (Christou, 1992; Birch, 1997; de la Riva *et al.*, 1998; Veluthambi *et al.*, 2003). *Agrobacterium*-mediated transformation has the advantages of low frequency of transgene rearrangement and simple segregation by low copy number (Sharma *et al.*, 2005). However, dicotyledonous species are generally more amenable to *Agrobacterium*-mediated transformation than monocots (Hagio, 1998). As taro is a monocot, biolistics would be a logical starting point in the development of a transformation system. Indeed, Fukino *et al* (2000) reported transformation in taro (*C. esculenta* var. *antiquorum*) by particle bombardment. However, the efficiency was not stated and although 96 bombardments were conducted, only two putative transgenic plants were analyzed. Consequently, in this research project the efficacy of both transformation methods will be examined with embryogenic suspension cultures of *Colocasia esculenta* var. *esculenta*.

### 2.3.3.1 Microprojectile bombardment

The particle bombardment method was developed by John Sanford and coworkers at Cornell University in the United States (Hagio, 1998). In this system (Figure 2.7), a suspension of DNA–coated tungsten powder (spherical particles 4.0 μm in diameter) was placed at one end of a bullet-like plastic macroprojectile. The macroprojectile was accelerated by a gunpowder charge and upon impact with a stopping plate at the end of the acceleration tube, the macroprojectile extrudes through a small orifice. This extrusion further accelerates the microprojectiles into the appropriately placed plant tissue. Although the gunpowder model was found to be successful for genetic transformation of various plant species, the lack of control over the force of bombardment as well the physical damage to target cells limited the number of stable transformations. The current model, PDS-1000/He$^{TM}$ device (Figure 2.8), is powered by a helium gas pressure breaking a rupture disc which then accelerates a macrocarrier, upon which DNA-coated microcarriers have been dried. Compared to the gunpowder device, it allows better control over bombardment parameter, distributes microcarriers more uniformly over target cells, is gentler to target cells, is more consistent between bombardments and yields several folds more transformations. Particle bombardment has proved to be the method of choice for several species mostly due to their recalcitrance to *Agrobacterium* and, as such, many food crops have been transformed using this technique (Hagio, 1998). In addition, it is robust in nature, relatively easy to operate and capable of producing reproducible results (Taylor and Fauquet, 2002). One of the disadvantages of particle bombardment is that it can result in high transgene copy number and a high frequency of transgene rearrangement, which may lead to transgene silencing or co-suppression (O'Kennedy *et al.*, 2001).

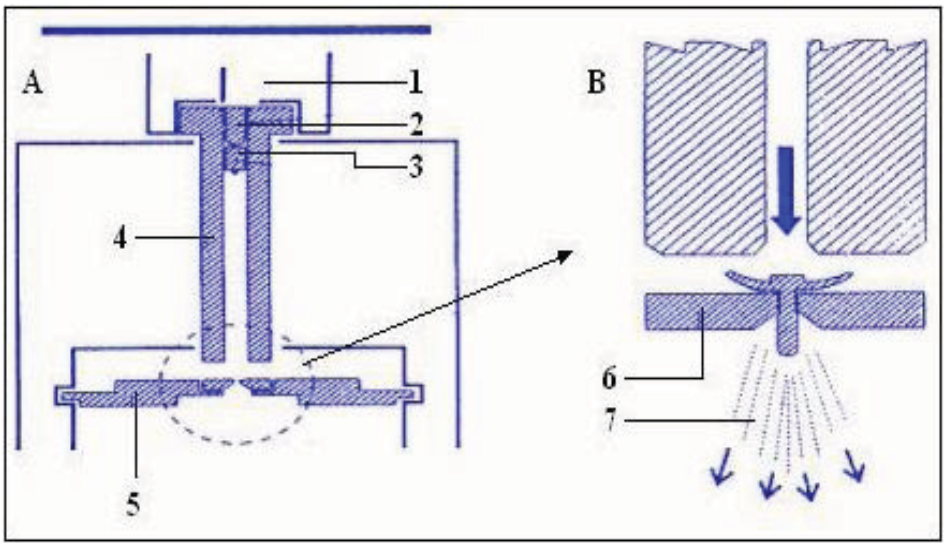


**Figure 2.7:** Schematic diagram of the gunpowder-driven particle gun.

(A) Before firing (B) After firing. 1-Firing pin, 2-Gunpowder charge, 3-Macrocarrier (plastic bullet) with microcarriers (tungsten particles), 4-Acceleration tube, 5-Stopping plate shelf with stopping plate, 6-Stopping plate with extruded macrocarrier (plastic bullet), 7-Launched microcarriers (tungsten particles) (Hagio, 1998)

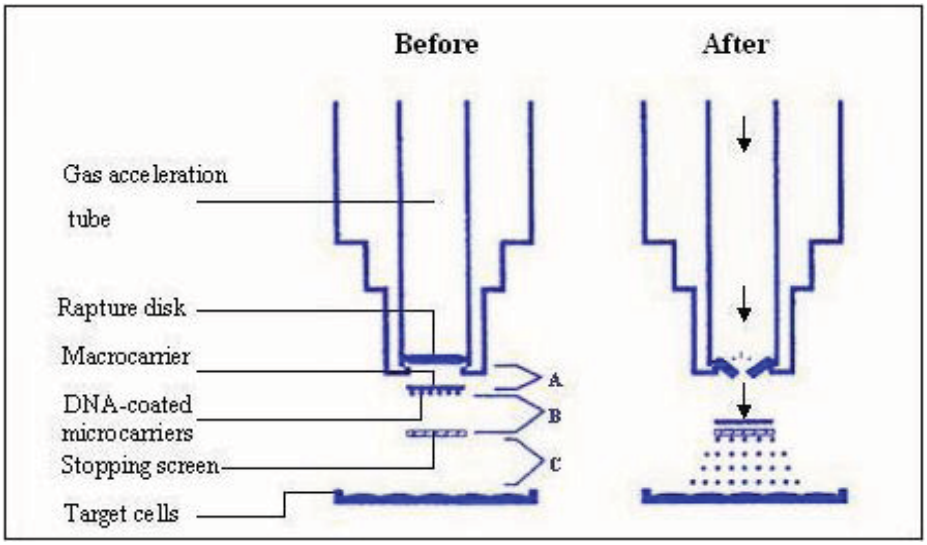


**Figure 2.8:** Schematic diagram of the helium-driven particle gun
(BIO RAD PDS-1000/He). The distance A, B and C can be changed (Hagio, 1998)

Regardless of the method employed, every stable transformation process demands the simultaneous occurrence of two independent biological events: the stable insertion of the transgene into the plant genome and regeneration from those cells in which this has occurred, producing a non-chimeric transgenic plant (Alves *et al.*, 1999).

#### *2.3.3.1.1 Parameters affecting biolistic transformation*

Although biolistic transformation eliminates the problem of species-dependency of *Agrobacterium* to plant cells, the method should not be regarded as species-independent (Chandra and Pental, 2003). Stable transformation of plants using biolistics requires the penetration of cells by microprojectiles, integration of the transgene of interest into the host plant genome followed by subsequent expression, and finally, continued growth of the transformed cells and regeneration of plantlets (Russell *et al.*, 1992). Different plant species may behave differently throughout any of these steps.

Several factors have been reported to affect the efficiency of particle and DNA delivery into the plant cells and subsequent transient expression and stable integration of the transgene. Microcarriers (or microprojectiles) may consist of different materials and be of different sizes. The two most commonly used microcarriers are gold and tungsten. In general, gold particles are preferred as they are more uniform in size and shape than tungsten resulting in less cell damage. Further, tungsten particles may undergo surface oxidation, which can alter DNA binding and catalytically degrade DNA bound to them (Sanford *et al.*, 1993) and cause toxicity problems in certain species (Russell *et al.*, 1992). Tungsten should not be immediately excluded as an option for biolistics as it is considerably less expensive and perfectly satisfactory for some applications.

Small changes in the diameter of the particle can have an impact on particle momentum, the quantity of DNA the particle carry and the size of the lesion produced in plant cells, hence affecting cell survival (Klein *et al.*, 1988; Häggman and Aronen, 1998; Janna *et al.*, 2006). There is a range of sizes available from 0.6 μm up to 1.6 μm. It has been demonstrated that transient GUS expression is much greater using

1.0 μm rather than 1.6 μm particles (Tian and Seguin, 2004). Particles smaller than 1.0 μm are usually reserved for small cells such as microalgae, yeast and bacteria.

Multiple bombardments of the same target tissue have been trialed in an attempt to transform a greater number of cells. This strategy has been shown to both increase (Klein *et al.*, 1988) and decrease (Janna *et al.*, 2006) transient expression. The decrease was most likely due to excessive damage.

Other biolistic parameters shown to affect transformation efficiency include helium pressure, macrocarrier flight distance to baffle screen, distance from baffle screen to target tissues and vacuum pressure. The factors which affect the delivery and transient expression of the gene have been studied and optimized for different species (Quoirin *et al.*, 1997; Marchant *et al.*, 1998; Deroles *et al.*, 2002; Janna *et al.*, 2006). It is apparent that optimal parameters for transformation efficiency must be arrived at empirically for each species.

Use of osmoticums such as mannitol, sorbitol, sucrose and myo-inositol, have also been reported to enhance biolistic gene transfer and subsequent reporter gene expression in some species. For example, Clapham *et al* (1995) reported a five to twelve fold increase in reporter gene expression in embryogenic cell cultures of *Picea abies*, which were treated with myo-inositol, before and after bombardment. Moreover, Ye *et al* (1990) also reported a 20-fold increase in transformation efficiency in chloroplasts using sorbitol and mannitol in the bombardment and incubation medium. It has been proposed that osmotic treatment causes cells to become plasmolysed and by reducing turgor pressure, extensive damage to cell membrane is minimized and the leakage of the protoplasm is prevented when the microcarriers penetrate the cells (Hagio, 1998; Marchant *et al.*, 1998; Santos *et al.*, 2002). In addition, since plasmolysed cells are less rigid, particle penetration may also be improved (Hagio, 1998). Osmoticums are not required for all species and for those where it is used, the type and concentration is variable (Hagio, 1998).

### 2.3.3.2 *Agrobacterium*-mediated transformation

*Agrobacterium tumefaciens* is a gram-negative soil bacterium, which causes crown gall disease in some plants species. They harbour large tumor inducing (Ti) plasmids of more than 200 kb in size, which contain the oncogenic, opine synthesis and virulence (*vir*) genes necessary for the establishment of infection and also opine catabolism genes allowing the bacterium to catabolise that particular set of opines (de la Riva *et al.*, 1998). In addition, Ti-plasmids also contain genes for conjugative transfer of plasmids between *Agrobacterium* (Figure 2.9) (Opabode, 2006). Ti-plasmids are classified as octopine, nopaline or agropine based on the type of opine produced and excreted by the tumors they induce. Thus, an *Agrobacterium* strain with an octopine Ti-plasmid will induce tumors that synthesize octopine and also encode genes required to utilize octopine as a source of carbon and nitrogen (Knauf *et al.*, 1983).

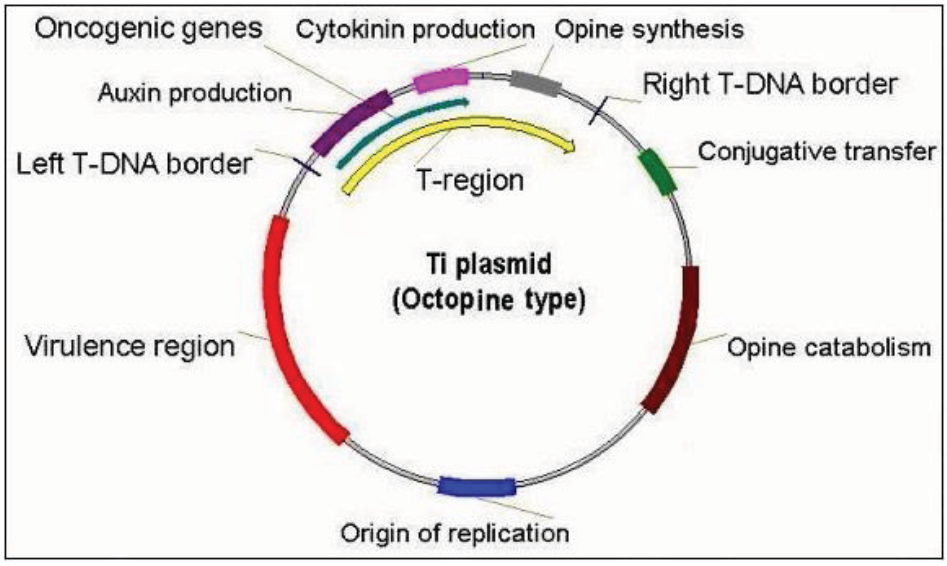


**Figure 2.9:** Diagrammatic representation of Ti-plasmid of *Agrobacterium tumefaciens*

(CAMBIA, 2003)

Within the Ti plasmid is a region that is copied and transferred to the plant cell, called the transfer or T-DNA. It contains two types of genes: the oncogenic genes encode enzymes involved in the synthesis of auxins and cytokinins which are responsible for tumor formation and the genes encoding enzymes for the synthesis of opines. Opines are exclusively utilized by *Agrobacterium* as a carbon and nitrogen source (Tzfira and Citovsky, 2006). The T-DNA region is flanked at each end by 25 bp T-DNA borders, the left and the right border, which are essential for T-DNA transfer. They are the target of Vir D1/Vir D2 border specific endonucleases, which process T-DNA from Ti-plasmid (Gelvin, 2003) and function in a *cis*-acting fashion (de la Riva *et al.*, 1998).

The virulence genes (*vir* genes) located on the Ti-plasmid encode a set of proteins responsible for excision, transfer and integration of T-DNA into the plant genome. The virulence region (30 kb) is organized in six operons: *vir* A, *vir* B, *vir* D and *vir* G are essential for the T-DNA transfer while *vir C* and *vir* E increase the efficiency of T-DNA transfer. The number of genes per operon differs: *vir* A, *vir* G and *vir* F have only one gene, *vir* E, *vir* C and *vir* H have two genes, *vir* D and *vir* B has four and eleven genes, respectively (de la Riva *et al.*, 1998).

The transformation process begins with bacterium-plant cell attachment, followed by the induction of *vir* gene expression (Figure 2.10). The Vir A protein functions as a sensory receptors and perceives the presence of plant phenolic compounds that are released upon wounding whereupon it autophosphorylates. Activated Vir A then transphosphorylates Vir G, which in turn interacts with *vir* promoter sequences and induces transcription of other *vir* genes (Gelvin, 2003). A single-stranded (ss)T-DNA molecule is then produced by the combined action of the bacterial Vir D1 and Vir D2 proteins. These proteins recognize the T-DNA border sequences and nick the bottom strand at each border. As a result, a single stranded T-DNA molecule is formed, which is transferred to the plant cell. After cleavage, Vir D2 remains attached to the 5'end of the ssT-strand. This association prevents the exonucleolytic attack of the 5'end of the ssT-strand and distinguishes the 5'end as the leading end of the T-DNA transfer complex. A Vir B/D4 type IV secretion system exports this complex along

with other Vir proteins to the host. Once inside the host-cell cytoplasm, the Vir E2 protein coats the ssT-DNA-Vir D2 complex. This association prevents the attack of nucleases and also reduces ssT-DNA secondary structure making the translocation through the membrane easier. Vir E2 and Vir D2 contain two and one plant nuclear location signals (NLS), respectively. Thus, both proteins mediate the uptake of T-DNA into the plant nucleus. The process of transport through the cytoplasm, nuclear import, intranuclear transport, T-DNA uncoating and integration into host genome utilizes the cellular machinery and mechanisms of the host and some bacterial Vir proteins (Vir D2, Vir E2 and Vir F) (de la Riva *et al.*, 1998; Gelvin, 2003; Tzfira and Citovsky, 2006). Integration of T-DNA into the host genome occurs via illegitimate (non-homologous) recombination (Tzfira *et al.*, 2004). Some of the *Agrobacterium* chromosomal genes are also involved in facilitating the transformation process by initiating: exopolysaccharide production, modification and secretion (*psc* A/*exo* C, *chv*A and *chv* B), bacterial attachment to plant cell (*att* genes), sugar transporters involved in coinduction of *vir* genes (*chv* E), regulation of *vir* gene induction (*chv* D) and T-DNA transport (*acv* B) (Gelvin, 2003).

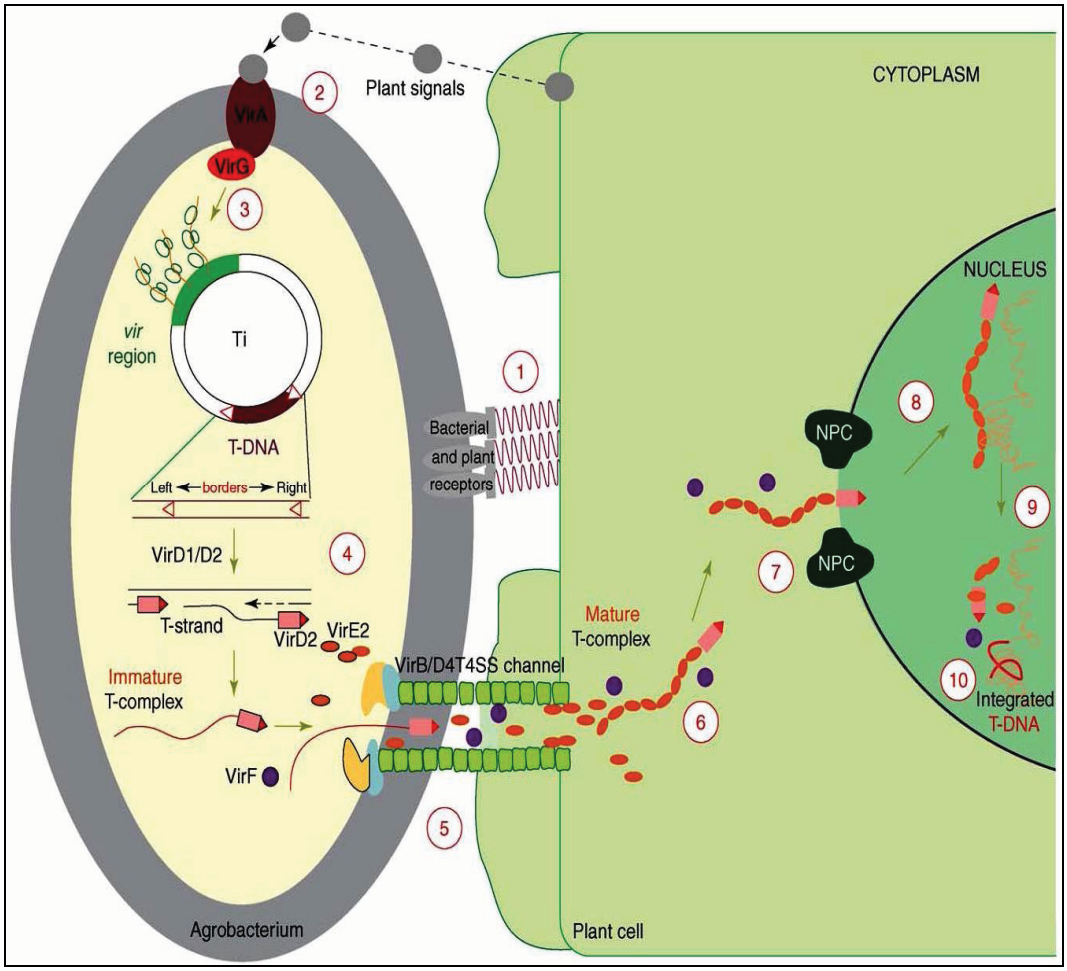


**Figure 2.10:** Model for *Agrobacterium*-mediated transformation of plants.

Transformation process begins with recognition and attachment of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal transduction system (2). Following activation of the *vir* gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2-DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10) (Tzfira and Citovsky, 2006)

#### *2.3.3.2.1 Using Agrobacterium as a tool for plant transformation*

Most of the protocols established to date for plant transformation via *Agrobacterium* have relied on the innovation of binary vectors and virulence helper Ti-plasmids (Figure 2.11) (Tzfira and Citovsky, 2006). The binary vector strategy is based on the fact that the *vir* genes and T-region could be separated into two different replicons. When these replicons are within the same *Agrobacterium* cell, the products of *vir* genes could operate in *trans* on the T-region to effect T-DNA processing and transfer to a plant cell provided the DNA is placed between two correctly oriented T-DNA borders. Therefore, the native T-DNA of the Ti-plasmid can be removed to prevent tumor formation (disarmed) to produce a virulence helper Ti-plasmid. The T-DNA is located on another smaller plasmid, the binary vector that contains multiple cloning site, markers for selection and maintenance in both *E. coli* and *Agrobacterium*, plant selectable marker gene between the right and left borders of T-DNA and origin of replication (ori) that permits the maintenance of plasmid in *E. coli* and *Agrobacterium*. In the binary Ti vectors, the plant selectable marker genes are placed near the left border (LB) while the gene of interest is placed near the right border (RB). Since during T-DNA transfer the RB precedes the LB, it could be that the selectable marker gene will be transferred first into the plant cell. As a result, transgenic plants containing only the selectable marker gene could be created if the bacterium to plant T-DNA transfer is interrupted. Hence, placing the gene of interest closer to the RB ensures that it will be transferred before the selectable marker gene (Hellens *et al.*, 2000). The binary vector can be cloned in *E. coli* and transformed into an *Agrobacterium* strain containing a helper plasmid (Gelvin, 2003).

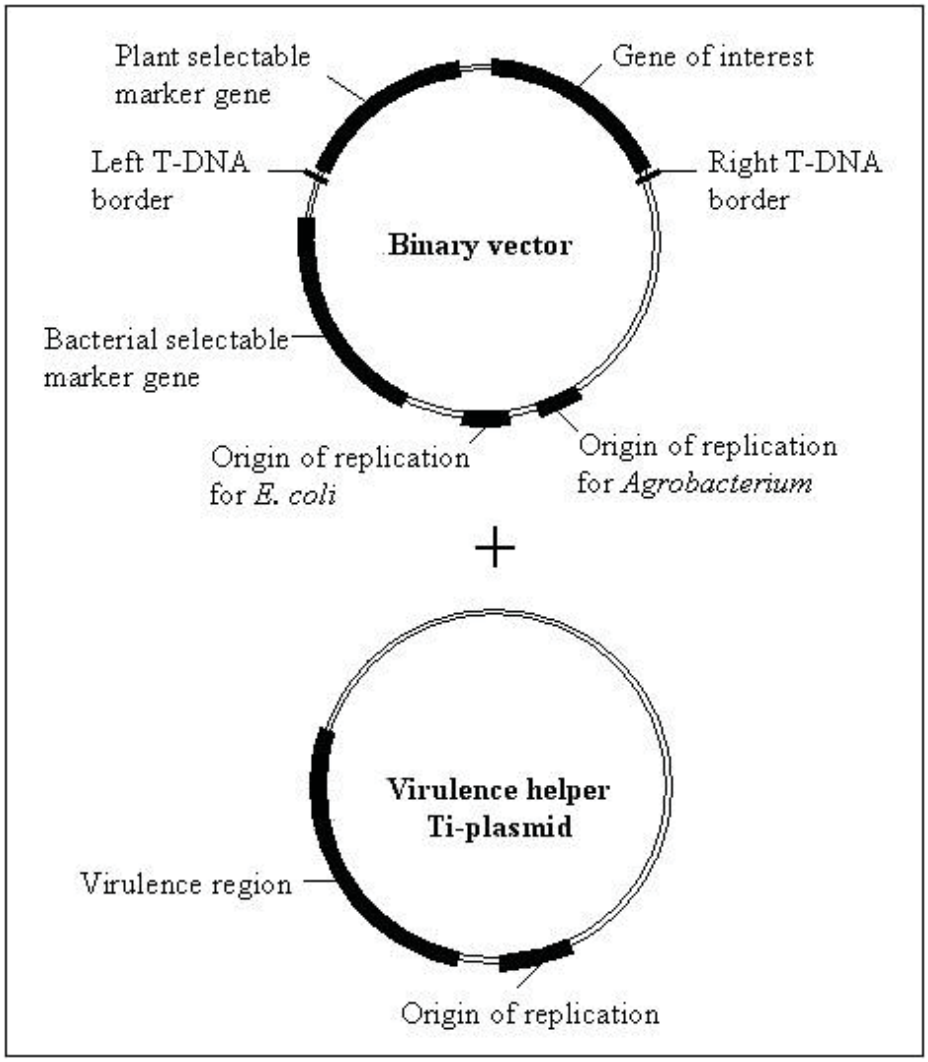


**Figure 2.11:** Diagram depicting the binary vector strategy used in plant transformation by *Agrobacterium tumefaciens*

Typical binary vector system consists of a small binary vector and a large virulence helper Ti-plasmid**.** The binary vector has a plant selectable marker gene and the transgene between the left and right borders. *E. coli* origin of replication allows initial cloning steps to be done in *E. coli. Agrobacterium* containing the virulence helper Ti-plasmid can be transformed with the binary vector and transformants selected using the bacterial marker gene on the binary vector. The virulence helper plasmid contains virulence genes necessary for T-DNA transfer to plant cells.

#### *2.3.3.2.2 Factors affecting Agrobacterium-mediated transformation*

The transfer of T-DNA and its stable integration into the plant genome is influenced by several factors. Plant tissues require an optimum density of *Agrobacterium* cells for a high frequency of transformation. In wheat inflorescence tissue, a cell density of less than 1.0 OD (optical density) resulted in very low transient GUS expression whereas at densities of 1.0-1.5 OD very high GUS expression was achieved (Amoah *et al.*, 2001). A cell density above 1.5 OD significantly reduced the frequency of transformation. In banana cell suspensions, although high inoculum densities (1.0 and 2.0) resulted in high transient gene expression, cell death was also high (Khanna *et al.*, 2004) indicating that densities giving high transient expression may not translate into a high number of stable transformants. In contrast to the two previous examples, use of a 0.5 OD of *Agrobacterium* resulted in severe necrosis in cauliflower (*Brassica olerecea* var. *botrytis*) explants, but a 1:20 dilution of the same culture significantly increased transient GUS expression (Chakrabarty *et al.*, 2002). It has also been demonstrated that the effect of *Agrobacterium* cell density on transformation efficiency can be manipulated by varying the duration of co-culture time, with high cell density and reduced co-culture time increasing the frequency of transformation (Amoah *et al.*, 2001; Opabode, 2006). Therefore, it is essential to optimize the inoculum levels of *Agrobacterium* so that cell necrosis is minimized while a high level of T-DNA transfer is maintained.

The use of certain compounds such as acetosyringone and pluronic acid F68 has been reported to increase the efficiency of transformation. Acetosyringone (a low molecular weight phenolic compound) is used as an exogenous stimulant for the induction of *vir* genes (Chakrabarty *et al.*, 2002; Opabode, 2006). Some monocotyledonous plants produce only very low levels or none of these types of phenolic compounds and thus cannot activate the vir genes of *Agrobacterium* (Suzuki *et al.*, 2001). Therefore, exogenous application of acetosyringone in co-cultivation medium has been recommended to overcome this problem. Incorporation of acetosyringone in the bacterial culture medium (pre-induction) and in the medium in which *Agrobacterium* is co-cultured (co-cultivation) with plant cells increased T-

DNA transfer into banana suspension cells (Khanna *et al.*, 2004) and in *Agapanthus praecox* ssp. *orientalis* (Suzuki *et al.*, 2001). There is a range of concentrations within which acetosyringone is effective; a minimum at which it is effective and a maximum at which it becomes bacteriostatic (Amoah *et al.*, 2001). Pluronic acid F68 is a surfactant. It facilitates *Agrobacterium*–plant cell attachment and also reduces the effect of substances that inhibit *Agrobacterium* attachment, thus enhances T-DNA delivery by *Agrobacterium* (Opabode, 2006).

Even though successful transformations have been achieved by *Agrobacterium* in recalcitrant monocotyledonous crops such as maize, rice, wheat, sorghum and banana, the frequency of transformation may still be quite low. It has been reported that cells become necrotic after being infected by the bacterium (Carvalho *et al.*, 2004). Rinsing co-cultured cells with timentin and incorporating it in the selection medium has been shown to be effective in eliminating *Agrobacterium* from plant cells (Carvalho *et al.*, 2004). In addition, allowing explants to grow without selection except against *Agrobacterium* after co-cultivation could help them recover from infection and thus reduce cell necrosis (Carvalho *et al.*, 2004; Bhalla and Singh, 2008). Further, providing heat shock to plant cells before co-culturing with *Agrobacterium* has been shown to increase the viability of cells, resulting in the recovery of large number of transgenic plants (Khanna *et al.*, 2004).

*Agrobacterium* strains differ in their ability to infect plants and transfer T-DNA (Suzuki *et al.*, 2001; Khanna *et al.*, 2004). For example, in sunflower (*Helianthus annuus* L.) genotypes, cv. Capella and SWSR2 inbred line, *Agrobacterium* strain, LBA4404 was more effective with the former cultivar while the strain GV3101 was effective with the latter cultivar (Mohamed *et al.*, 2004). In cauliflower (*Brassica oleracea* var. *botrytis*), a high level of GUS expression was observed in explants infected with *Agrobacterium* strain GV2260, while co-cultivation with LBA4404 strain resulted in very low levels of expression (Chakrabarty *et al.*, 2002). Generally, the use of a highly virulent strain of *Agrobacterium* or using super-binary vectors enhances the frequency of transformation in recalcitrant crops (de la Riva *et al.*, 1998).

The type of explants (target material) is also important. *Agrobacterium* require cells that are actively dividing for gene transfer to occur (Okada *et al.*, 1986). Therefore, embryogenic callus and embryogenic suspension cells of monocots are frequently used as a suitable target. Like any method for the production of transgenic plants the target material must also be suitable for regeneration so that transgenic plants can be recovered (de la Riva *et al.*, 1998).

### 2.3.4 Regulatory sequences for transgene expression

Promoters are an essential element in transformation, as they are required to drive expression of both the selectable marker gene and the gene of interest. Numerous promoters are currently available, the most common of which are listed in Table 2.7.

Since high levels of expression are frequently desirable, constitutive promoters are commonly used. Constitutive promoters cause gene expression throughout the life of the plant in most tissues. The most widely used constitutive promoter is CaMV 35S derived from cauliflower mosaic virus (Alves *et al.*, 1999). Häggman and Aronen (1998) reported very high transient GUS expression in Scots pine embryogenic cultures using the constitutive CaMV 35S promoter. Similar results have also been reported by Zipf *et al* (2001). Even though a high level of transient expression is not the sole determining factor for stable transformation, it is a useful indicator for the development of stable transformation for many species (Tian and Seguin, 2004).

However, Häggman and Aronen (1998) argued that the effects of promoters are dependent on both the tissue type and the species. Consequently, for successful expression of the gene of interest in the target tissue/plant, a thorough and careful selection of a suitable promoter is essential (Zipf *et al.*, 2001).

**Table 2.7:** List of promoters used in transformation systems.

| Promoter | Derivation † | Specificity ‡ | Reference |
|---|---|---|---|
| *nos* | *Agrobacterium* nopaline synthase | Developmentally regulated, organ specific | (An *et al.*, 1988) |
| *CaMV 35S* | *Cauliflower mosaic virus* encoding 35S RNA | Constitutive | (Benfey *et al.*, 1990) |
| *Ubi*-1 | Maize polyubiquitin | Strongest in meristematic and vascular tissue, activity increased by heat shock | (Christensen *et al.*, 1992) |
| *rbcS* | Rubisco small subunit (from several monocot and dicot species) | Light induced expression in leaves | (Sugita *et al.*, 1987; Kyozuka *et al.*, 1993) |
| *Act* | Rice actin | Constitutive | (Zhang *et al.*, 1991) |

† Derivation: the organism and protein whose corresponding gene the promoter was derived from.
‡ Specificity: tissue in which promoter is active and physiological requirements for activation if applicable.

### 2.3.5 Marker and reporter genes are essential in transformation

When transformation protocols are developed, a selectable marker gene and a reporter gene are included in the transformation vector (Sharma *et al.*, 2005). After development of the protocol, the reporter gene is replaced with the gene of interest. Selectable markers consist of genes encoding enzymes capable of inactivating a toxic substance, commonly an antibiotic or herbicide. Alternatively, the toxic substance targets the active site of a protein vital for cellular process and the selectable marker gene codes for a variant of this vital protein, which is not affected by the toxin (Weeks *et al.*, 2000). Thus, selectable markers allow survival of the few cells in which the transgene has integrated facilitating efficient selection of transformed cells (Rao

and Rohini, 2003; Sharma *et al.*, 2005). The most commonly used selectable marker genes are: *npt II* gene (neomycin phosphotransferase II), which confers resistance to kanamycin or G 418 (geneticin), *hph* gene (hygromycin phosphotransferase) that confers resistance to hygromycin B, and *bar* gene (phosphinothricin acetyltransferase) that confers resistance to the herbicide phosphinothricin (Rao and Rohini, 2003).

For each transformation and regeneration system, a minimum level of a selective agent, which can fully inhibit the growth of non-transformed cells, should be determined by use of a "kill curve" (Yang *et al.*, 1999). A concentration of selective agent greater than that necessary is likely to result in reduced transformation efficiency. While transgenic cells might be resistant to the selective agent, it can sometimes be the case that the selective agent or its metabolite interferes with regeneration.

Positive selection has been proposed as a means of avoiding interference with regeneration by toxic agents and also removing the stigma of having antibiotic and herbicide resistance genes released into the environment (Yoo *et al.*, 2005). Positive selection relies on providing an essential nutrient in a form that can only by metabolized by the protein encoded by the marker gene. For example, the phosphomannose isomerase (PMI) system which uses mannose as the selective agent. Transgenic plants expressing the enzyme PMI encoded by the *man* A gene from *E. coli* are able to convert mannose-6-phosphate to fructose-6-phosphate which is metabolized through glycolysis (Joersbo, 2001; Reed *et al.*, 2001; Penna *et al.*, 2002).

Reporter genes are included in transformation vectors for two reasons: (i) to enable easy identification of potential transformants during the development of a transformation protocol and (ii) as a means of assessing tissue specificity and quantifying activity of promoters or other transgene systems. Reporter genes such as chloramphenicol acetyltransferase (CAT), β-glucuronidase (GUS), nopaline synthase and octopine synthase are bacterial in origin (Sharma *et al.*, 2005), while others have been derived from insects (luciferase, LUX) and jellyfish (green fluorescent protein, GFP). The most commonly utilized reporter gene is *uid* A, which encodes for the

enzyme β-glucuronidase (GUS). In the presence of the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), GUS cleaves glucuronic acid producing a soluble colourless indoxyl group which is rapidly oxidized and dimerizes to produce an insoluble indigo precipitate. GUS is easily visualized and the enzyme is relatively stable. However, the assay is destructive and GUS-positive explants cannot be recovered (Alves *et al.*, 1999). The "green fluorescent protein" (GFP) is another useful reporter gene for plant and animal systems (Alves *et al.*, 1999). The gene encoding GFP was originally derived from a bioluminescent jellyfish, *Aequorea victoria*. The coding region was modified such that the protein is able to emit green fluorescence upon excitation under blue or UV light without any additional substrate. It is also a non-destructive assay (Miki and McHugh, 2004).

### 2.3.6 Combining somatic embryogenesis and transformation for crop improvement

Efficient plant transformation systems require target tissue that is competent for proliferation and regeneration into plantlets. Direct organogenesis from the mature organs of monocotyledons and many dicotyledons occurs infrequently if at all. In most instances, a large population of totipotent cells in the form of callus is multiplied prior to transformation (Koichi *et al.*, 2002). Embryogenic cultures are the most commonly used target tissue for high frequency recovery of non-chimeric transgenic plants (Taylor and Fauquet, 2002). Such cultures possess a high proportion of cytoplasmically rich, actively dividing cells, which provide high levels of transient expression and high frequency transgene integration (Mahn *et al.*, 1995). In addition, it is believed that these cells are better able to overcome the stress induced by transformation (Santos *et al.*, 2002).

Somatic embryogenesis and genetic transformation are extremely valuable tools in plant biotechnology. Efficient embryogenic systems facilitate the generation of large numbers of transgenic lines required for screening the desired trait. It is common for commercial plant biotechnology companies to produce 200 transgenic lines all containing the same transgene in order to select one line, which has the new desired trait while retaining the superior agronomic traits of the parent plant. A selection of

plants which have been transformed and regenerated via somatic embryogenesis system are shown in Table 2.8.

**Table 2.8**: List of transgenic crops with agronomic traits regenerated via somatic embryogenesis.

| Crops | Agronomic traits introduced | Transformation methods used | References |
|---|---|---|---|
| Peanut | Tomato spotted wilt virus (TSWV) resistance<br>Sclerotinia blight resistance | Biolistics<br>Biolistics | (Parrott, 1999; 2000)<br>(Livingstone *et al.*, 2005) |
| Banana | Banana bunchy top virus resistance | Biolistics | (Becker *et al.*, 2000) |
| Rice | Blight resistance<br>Insect resistance | Biolistics<br>Biolistic | (IRRI, 1996)<br>(Alam *et al.*, 1998; Riaz *et al.*, 2006) |
| Soybean | Enhanced protein content (milk β-casein) | Biolistics | (Maughan *et al.*, 1999) |
| Cassava | Altered starch level (sweet cassava) | Biolistics | (Munyikwa *et al.*, 1998) |
| Wheat | Abiotic stress resistance (drought and salt resistance) | Biolistics | (Pauk *et al.*, 2002) |
| Cotton | High stearic and oleic acid in cotton seed oil | *Agrobacterium* | (Liu *et al.*, 2002) |
| Sugarcane | Sorghum mosaic virus (SrMV) resistance | Biolistics | (Ingelbrecht *et al.*, 1999) |
| Carrot | Salt tolerance | Biolistic | (Kumar *et al.*, 2004) |
| Lupin | Enhanced methionine levels in seeds | *Agrobacterium* | (Molvig *et al.*, 1997) |

As taro production is severely hampered by pest and disease problems, the availability of an efficient *in vitro* regeneration technique, which in turn provides a system for transformation, could provide a significant boost to taro production in the Pacific. The regeneration of transgenic taro through somatic embryogenesis with introduced fungus-resistant genes against TLB for instance, would be of significant benefit for taro growers throughout the region. Consequently, this project will look at the possibility of developing an embryogenic regeneration and transformation system for *Colocasia esculenta* var. *esculenta*.

# CHAPTER 3

## General Materials and Methods

### *3.1 Plant Material*

#### 3.1.1 Culture media and conditions

Unless otherwise stated, the culture media and conditions were as follows: non-heat labile components were added and the pH of culture media was adjusted to 5.8 with 1 M NaOH or HCl. Gelling agent was then added and the complete media melted on a hotplate. Media was then dispensed into culture vessels and autoclaved at 121 $^{o}$C, 15 lbs pressure for 15 minutes. If a heat labile substance was required, it was filter sterilized and added to autoclaved media after it had cooled to 40-50 $^{o}$C. Cultures were incubated at 25 $^{o}$C ± 2 $^{o}$C with a 16 hour photoperiod. Light was provided by cool white fluorescent lamps providing a photon flux density (PFD) of 30 μMol $m^{-2}$ $s^{-1}$.

#### 3.1.2 Selection and multiplication of axenic, virus-free plant material

#### 3.1.2.1 Virus indexing

Taro tissue culture accessions were initiated at the Centre for Pacific Crops and Trees (CePaCT) of the Secretariat of the Pacific Community (SPC) in Fiji. Once a shoot culture derived from a single meristem had grown and multiplied, clones of that accession were sent to the Queensland University of Technology, Brisbane, Australia for virus indexing. Accessions were tested for the presence of taro bacilliform virus (TaBV), dasheen mosaic virus (DsMV), taro reovirus (TaRV), Colocasia bobone disease virus (CBDV) and taro vein chlorosis virus (TaVCV). Plants were tested twice at three and six months and only accessions testing negative were used for further multiplication.

#### 3.1.2.2 Screening for endogenous bacteria

Virus-free accessions selected for bacterial screening were the esculenta varieties THA-07, CK-07 and CPUK, the former being derived from Thailand and the latter two from the Cook Islands. Taro tops were prepared from *in vitro* plants by removing the top ~0.5 cm of the corms and removing the leaves from the tops leaving ~1 cm of the petioles attached. The cut base of the tops were streaked on Petri dishes containing 523 medium (Reed and Buckley, 1999), then individual tops were transferred to 10 mL of solid MS medium in 28 mL McCartney bottles. Both Petri dishes and McCartney bottles were incubated as described above. After five days, cultures were checked for bacterial growth. The plant tops whose streak plate showed no bacterial growth on 523 medium were selected and multiplied.

### 3.1.3 Multiplication of plant material

Axenic, virus-free accessions were multiplied following the standard procedure used by the SPC-Centre for Pacific Crops and Trees (Taylor, pers. comm.). Medium consisted of Murashige and Skoog (1962) medium with 30 g/L sucrose and 7.75 g/L agar. Plantlets were cultured in 28 mL McCartney glass bottles containing 10 mL of media. Multiplication was in three stages: (I) 0.5 mg/L TDZ for four weeks, (II) 0.8 mg/L BAP for three weeks and (III) 0.005 mg/L TDZ for three weeks. Following multiplication, small suckers were allowed to develop into larger plantlets by first culturing individual suckers in hormone-free liquid MS medium for two to four weeks followed by culturing in agar-solidified MS medium for 12 to 16 weeks. For both these treatments, plantlets were cultured in 125 mL glass jars containing 50 mL of medium.

## *3.2 Callus Initiation, Proliferation and Regeneration*

### 3.2.1 Preparation of explants

Tissue culture plantlets were removed from culture bottles and the leaves removed by cutting the petioles just above the corm. The corms were then placed in sterile Petri dishes set under a dissecting microscope and debris scraped off gently from the corm surface using the blunt side of the scalpel blade. Transverse sections 1-2 mm thick were then cut.

### 3.2.2 Callus initiation

Callus was initiated in two stages. Initially, corm slices were maintained on half-strength MS medium containing 2,4-D for a short period of time (days) followed by subculture on half-strength MS containing TDZ. Cultures were maintained in the dark throughout the callus initiation phases.

### 3.2.3 Callus growth and maintenance

Corm-derived callus was proliferated using two callus maintenance media (CMM): (i) agar-solidified half-strength MS medium containing 800 mg/L glutamine and (ii) liquid half-strength MS medium containing 100 mg/L glutamine. In both CMM, the concentrations of TDZ and 2,4-D were 1.0 mg/L and 0.5 mg/L, respectively. Callus maintained on solid CMM was subcultured monthly, while suspension cultures were subcultured on a weekly basis. All cultures were maintained in the dark.

### 3.2.4 Regeneration

Regeneration from callus on solid medium was induced by transferring from solid CMM onto hormone free half-strength MS medium. The cultures were kept in low light intensity (5 μmoles photons.$m^{-2}$s). Regeneration from suspension cells was induced by plating cells on embryogenesis medium (EM) for somatic embryo initiation and maturation followed by transfer to germination medium (GM) then plantlet development on hormone free half-strength MS medium. The cultures were maintained in the dark during the initiation and maturation phases but exposed to low light intensity during the germination and plantlet development phases.

### 3.2.5 Histology

Embryogenic callus and putative somatic embryos were fixed in formaldehyde: alcohol: acetic acid (FAA) (1:1:8 v/v) for four days, dehydrated in a xylene and ethanol series, then infiltrated and embedded with paraplast and wax respectively. Six-micrometer thick sections were cut using a rotary microtome. The sections were heat fixed to 3-aminopropyltriethoxysilane (APES)-coated glass slides to improve tissue adherence. After fixing, the tissues were dewaxed and stained with either Ehrlich's HX and Eosin or Safranin O-Fast Green and observed under compound microscope (Olympus BX41).

## *3.3 Genetic Transformation of Taro*

The target material for both *Agrobacterium* and biolistic transformation was embryogenic suspension cell aggregates sieved through a stainless steel sieve (500 μm diameter). The methods developed for Cavendish banana (*Musa* spp. AAA group) transformation by Becker *et al* (2000) and Khanna *et al* (2004) served as a starting point for the development of microprojectile particle bombardment and *Agrobacterium*-mediated gene transfer, respectively. These were modified as necessary.

## *3.4 Statistical Analysis*

Data were analyzed by analysis of variance (ANOVA) and t-tests wherever appropriate using a 95 % confidence interval. Where $p<0.05$, significant differences between individual treatment means were determined using Fisher's Least Significant Difference (LSD) test. All data were analyzed by SPSS for windows, version 11.

# CHAPTER 4

## Factors Affecting the Initiation of Embryogenic Callus in Taro (*Colocasia esculenta* var. *esculenta*)

### *4.1 Introduction*

Embryogenic callus induction is a vital stage for indirect somatic embryogenesis. Although many embryos can be produced from callus derived from the original explant, embryo production can be greatly increased if the callus can be proliferated on solid or liquid medium. Subsequent somatic embryos often require a maturation phase before germination into plantlets. The potentially large numbers of embryos which can be produced make the system attractive for micropropagation while their unicellular origin make them ideal target tissue for the production of non-chimeric transgenic plants (Vasil and Vasil, 1981). Further, the primary somatic embryos can be used to generate secondary somatic embryos which, like the primary embryo, are also unicellular in origin (Hu and Khalil, 2004).

Embryogenic callus and subsequent somatic embryos have been obtained from various tissue types of both *in vitro* and *in vivo* origin. The use of *in vitro* plant material has the advantage of explants being generally free from fungus and exogenous bacteria and thus not requiring lengthy decontamination procedures. It also ensures a continuous supply of axenic tissue throughout the year irrespective of seasonal influences. Regardless of the tissue source, younger/less differentiated tissues are generally preferred since they frequently contain meristematic cells which readily form embryogenic callus (Williams and Collins, 1976).

During callus formation, cells within explants undergo dedifferentiation to produce a mass of undifferentiated cells (callus). One of the consequences of the dedifferentiation process is that most of the explants lose the ability to

photosynthesize. Further, due to physical isolation from the donor mother plant their metabolic profile does not match that of the donor plant (Slater *et al.*, 2003). This necessitates the manipulation of the nutrient components (Slater *et al.*, 2003) and the application of appropriate growth regulator(s) (Jiménez, 2001) in the culture media. For example, in wheat the frequency of embryogenic callus was increased when the concentration of macronutrients was increased from half to full or double-strength in initiation medium containing 2,4-D (Haliloglu, 2002), while half-strength MS medium containing TDZ favoured the initiation of embryogenic callus in banana (Srangsam and Kanchanapoom, 2003).

The selection of a suitable explant, the determination of the optimal concentrations of various growth regulators and culture conditions are equally important for somatic embryogenesis to occur. These factors have been shown to significantly affect somatic embryogenesis in many species (Oka *et al.*, 1995; Haggman *et al.*, 1999; Zhang *et al.*, 2001; Kim, Park *et al.*, 2004; Wu *et al.*, 2004). The chemical stimuli in the culture medium and the physical conditions such as light and temperature trigger the meristematic cells within the explant to embark on an embryogenic path.

In taro (*Colocasia esculenta* var. *esculenta*), various types of explants have been used to initiate organogenic callus (Abo El-Nil and Zettler, 1976; Jackson *et al.*, 1977; Yam *et al.*, 1990). However, the use of corm explants for the initiation of embryogenic or organogenic callus and indirect somatic embryogenesis (that is, through an intervening callus phase) has not been reported to date in either of the taro varieties, *esculenta* and *antiquorum*. Since taro is an important subsistence and trade crop, an efficient somatic embryogenesis system would not only generate substantial numbers of plantlets for propagation, but also provide suitable target tissue for genetic transformation. This in turn could potentially provide a solution to many devastating pests and diseases threatening taro production in the Pacific through the generation of resistant plants.

The aim of the research in this chapter was to examine the factors affecting the initiation of embryogenic callus in *Colocasia esculenta* var. *esculenta*. The specific

objectives of this study were to (i) identify a suitable explant, (ii) determine the optimal concentration of PGRs and inorganic salts and vitamins and (iii) study the effect of genotype on somatic embryogenesis in taro.

## *4.2 Materials and Methods*

### 4.2.1 Culture medium for callus initiation

The medium used for callus initiation and proliferation consisted of half-strength MS medium, 30 g/L sucrose, 7 g/L agar and various concentrations of the growth regulators, 2,4-D and TDZ. Explants were cultured in 90 x 15 mm Petri dishes containing 25 mL of medium.

### 4.2.2 Preparation of corm explants

Leaves, leaf bases and roots were removed from tissue culture plantlets to expose only the corm. Transverse sections 1-2 mm thick were then cut and were placed on callus initiation medium.

### 4.2.3 Callus initiation – stage I

Corm slices were first placed on callus initiation medium-stage I (CIM-I), which consisted of medium as described above with various concentrations of 2,4-D (0, 1.0, 2.0, 4.0 mg/L). The explants were placed horizontally with the lower cut surface (in relation to its original orientation within the corm) in contact with the medium and cultured in the dark for 20 days. In one experiment, the initial incubation time on CIM-I was examined by culturing for 10, 20 or 40 days using 2.0 mg/L 2,4-D only.

### 4.2.4 Callus initiation – stage II

After CIM-I treatment, explants were transferred to callus initiation medium-stage II (CIM-II), which consisted of the medium described above with various concentrations of TDZ (0, 0.25, 0.5, 1.0, 2.0 mg/L). The explants were placed on the culture medium in the same manner as in CIM-I and continued to be cultured in the dark. The combinations of growth regulator treatments used in CIM-I and CIM-II are shown in Tables 1 and 2.

### 4.2.5 Effect of medium concentration on callus initiation

The effect of medium concentration on callus initiation was examined using quarter, half and full-strength MS medium. The concentrations of 2,4-D and TDZ in CIM-I and II were 2.0 mg/L and 1.0 mg/L, respectively. Explants were maintained on CIM-I for 20 days and then subcultured onto CIM-II. The experimental conditions were the same as above.

### 4.2.6 Effect of genotype on callus initiation

The effect of genotypes on callus initiation was examined using the following cultivars: CPUK, CK-07 and THA-07. The basal medium used was half-strength MS and concentrations of 2,4-D and TDZ in CIM-I and CIM-II were as above. The explants were treated in the same manner with the same culture conditions as above.

For each combination of treatments there were 10 replicate plates, with each plate containing 10-16 explants. All cultures were observed fortnightly and the final results were recorded 100 days after initiating the cultures. Data was recorded as the percentage explants per Petri dish producing callus and the percentage callus-producing explants undergoing regeneration via either embryogenesis or organogenesis. Mean values for each treatment were derived from the 10 replicate Petri dishes.

### 4.2.7 Histology

Embryogenic tissue was placed in formaldehyde-glacial acetic acid-ethanol (FAA): 1:1:8 (v/v) for at least four days to allow the fixative to penetrate the tissue. Fixed tissues were then processed overnight in an automatic wax tissue processing machine (Tissue-Tek VIP, Miles Scientific) on a 16 h cycle at the following settings: (i) tissue dehydration; 70 % ethanol for 1 h followed by two 1 h 90 % ethanol cycles, two 1 h 100 % ethanol cycles and one 2 h 100 % ethanol cycle, (ii) tissue clearing; 100 % xylene for 1 h followed by two 2 h cycles in 100 % xylene and (iii) infiltration of paraplast; four 1 h cycles in paraplast. Processed embryogenic tissues were then embedded in paraplast blocks (Tissue-Tek uni-cassette, Miles Inc. 4173). The

prepared blocks were trimmed and 6 µm thick sections were cut using a series '820' Spencer rotary microtome.

The sections were mounted on slides and then dried overnight at 37 $^{o}$C. After fixing them to slides at 60 $^{o}$C for 30 minutes, they were dewaxed in 100 % xylene for 10 minutes and rehydrated by dipping twice in 100 % ethanol followed by 90 % ethanol, 70 % ethanol and then one wash in tap water. Hydrated sections were then stained with either Ehrlich's HX-Eosin or Safranin-O Fast Green to detect proteins, starch, cell wall and nuclei.

For Ehrlich's HX-Eosin staining, dewaxed and rehydrated sections were first stained with Ehrlich's HX solution (10 min), rinsed in tap water (10 min), differentiated in acid alcohol (4 dips), rinsed in tap water, dipped in 1 % ammonia solution for 30 seconds followed by rinsing in tap water. The sections were then dipped in 70 % and 90 % alcohol, respectively, and counterstained with Eosin (3 min). The stained sections were then dehydrated by quickly dipping in 90 % ethanol followed by absolute alcohol and 100 % xylene.

For Safranin-O Fast Green staining, dewaxed and rehydrated sections were stained in Fast Green (FCF) solution (0.001 %) for five minutes, then rinsed quickly with 1 % acetic acid solution (10-15 seconds only). After acid treatment, the sections were stained in 0.1 % Safranin O solution (5 min) then dehydrated and cleared with 90 % ethyl alcohol, absolute alcohol and xylene changes (2 minutes for each change).

Stained sections were mounted in Depex (BDH, 361254D), covered with a glass slip and viewed using a Leica (Laborlux-S) compound light microscope at 40-1000 times magnification.

## *4.3 Results*

### 4.3.1 Effect of explants type, light and dark treatment on callus initiation

Preliminary experiments were conducted to determine the effect of light and dark on callus initiation using shoot-tips, third and fourth leaf primordia and petiole explants and half-strength MS medium containing various concentrations of 2,4-D (0, 0.5, 1.0 2.0 mg/L) and incubating both in light and dark. Three replicates were taken for each culture regime with 5-10 explants per hormonal treatment. After one month, a very low frequency (2-3 petiole and 1-2 shoot-tip explants per replicate) of non-regenerable callus was observed on the basal part of petioles from third leaf primordia and shoot-tip explants cultured on 1.0 mg/L 2,4-D maintained in the dark while no callus formed on explants maintained in light (Table 4.1; Figure 4.1A-C). The frequency of callus initiation was increased when petiole explants from third leaf primordia were incubated on 2,4-D for various durations (3, 6, 9, 12 and 15 days) followed by transfer on TDZ. Substituting the petioles with corm sections as explants and increasing 2,4-D incubation time to 20 days resulted in a dramatic increase in callus formation. Based on these initial observations, all subsequent experiments on embryogenic callus initiation were conducted and optimized using this protocol, corm explants and dark treatment.

**Table 4.1:** The effect of light and dark treatment on callus initiation from different explants in taro cv. CPUK. The explants were cultured on half-strength MS medium containing various concentrations of 2,4-D and kept in either light or darkness for callus induction.

| Explant Type | 2,4-D (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Light Treatment | | | | Dark Treatment | | | |
| | 0 | 0.5 | 1 | 2 | 0 | 0.5 | 1 | 2 |
| Shoot-tip | - | - | - | - | - | - | + | - |
| 3rd Leaf primordia | - | - | - | - | - | - | - | - |
| 3rd Petiole | - | - | - | - | - | - | ++ | - |
| 4th Leaf primordia | - | - | - | - | - | - | - | - |
| 4th Petiole | - | - | - | - | - | - | - | - |

Results are derived from three replicates with 5-10 explants per replicate.
No callus formation (-), 1-2 explants forming callus (+), more than two explants forming callus (++).

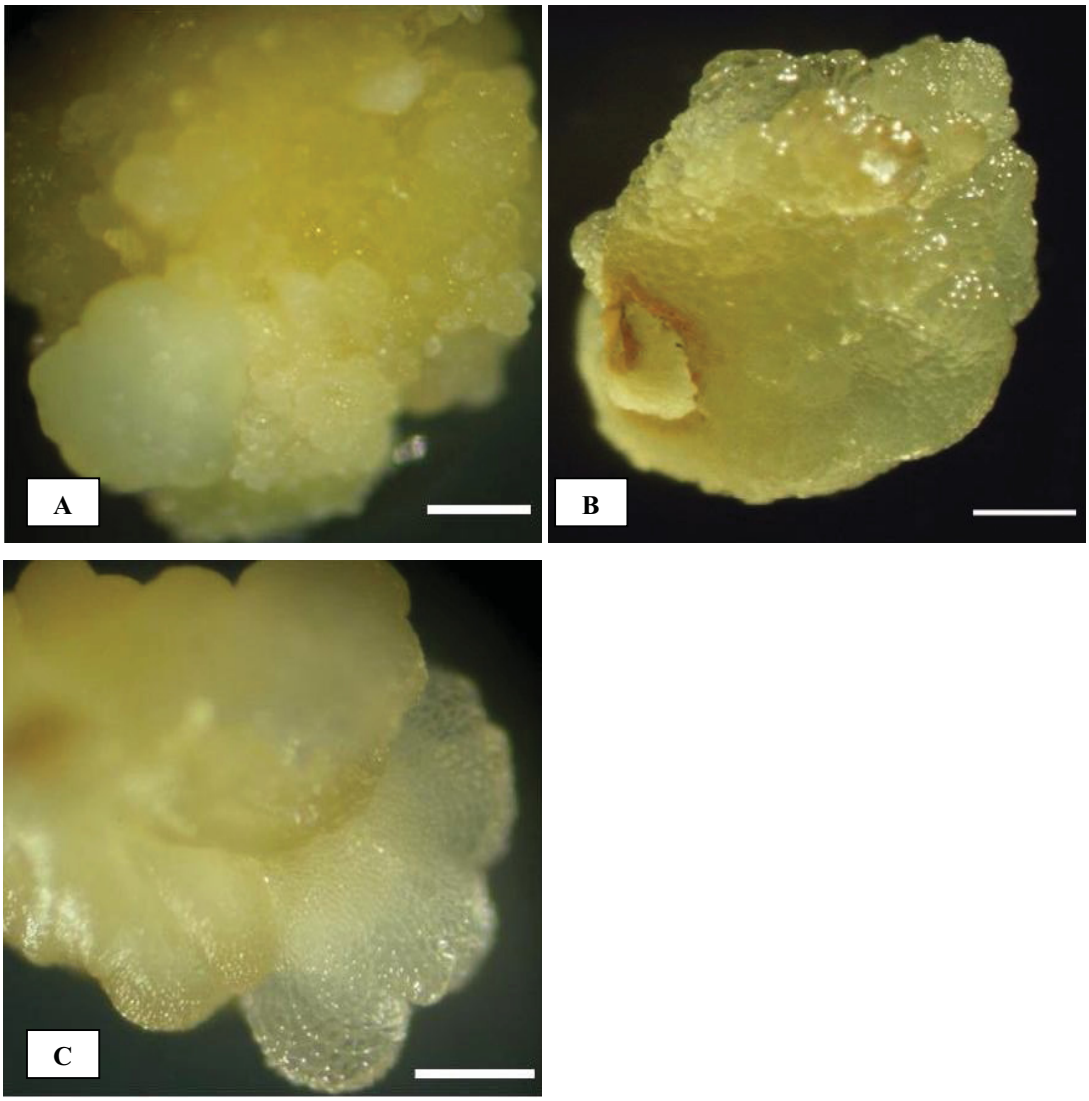


**Figure 4.1:** Callus formation in petiole and shoot-tip explants of taro cv. CPUK in dark culture. Non-regenerable callus began to form after one month on third leaf petiole (A, B) and shoot-tip (C) explants cultured on 1 mg/L 2,4-D. Scale bar = 2 mm.

### 4.3.2 Effect of 2,4-D and TDZ on callus initiation

The effect of various 2,4-D and TDZ concentrations on callus formation and regeneration was examined. Callus was initiated from corm explants in two stages: initially explants were cultured on half-strength MS medium containing various concentrations of 2,4-D for 20 days followed by transfer to various concentrations of TDZ. Transfer of the explants from medium containing 2,4-D to medium without TDZ or from medium without 2,4-D to medium containing TDZ resulted in the formation of non-regenerable callus as indicated by the lack of differentiation. Regenerable callus only formed when explants were cultured on media containing 2,4-D followed by media containing TDZ (Table 4.2). Explants receiving no hormone treatment did not form any callus and turned necrotic after 30-40 days.

After 15 days on all 2,4-D concentrations, approximately 50 % of the explants swelled in the mid portion, after which a soft watery callus developed (Figure 4.2A). Upon transfer to all concentrations of TDZ, callus formation became more rapid and was friable, cream or white in colour and cells were large and vacuolated (Figure 4.2B). After 45 days on TDZ (0.5 and 1.0 mg/L), structures resembling adventitious shoots began appearing on white compact callus (Figure 4.2C) while, what appeared to be somatic embryos, began appearing after 75 days on yellow nodular callus (Figure 4.2D). Initially, only very few (2-3) explants formed embryogenic callus but by 100 days the frequency was greater and a few explants (2) on higher TDZ (2.0 mg/L) also produced the callus. Callus was classified as either embryogenic or organogenic based on morphology and the structures emanating from them. Embryogenic callus was characterized by appearing glossy, compact, and pale yellow-cream in colour and producing translucent globular structures. Histological analysis of embryogenic callus revealed the presence of two cell types: i) embryogenic cells, which were small, isodiametric, contained a large nucleus and had dense cytoplasm and ii) non-embryogenic cells, which were large vacuolated parenchymal cells containing few plastids. The embryogenic cells were generally located on the periphery of the callus and had a clustered distribution, interspersed with large parenchymal cells (Figure 4.3A and 4.3B). The embryo-like structures

contained both shoot and root meristems (Figure 4.4A-C). Organogenic callus was characterized by being glossy, compact, and white in colour and producing elongated opaque structures. Histological analysis of this type of callus was not done. In general, individual explants produced both types of callus. After approximately 110 days on TDZ, there was no further development of either organogenic or embryogenic callus. However, when maintained on the same medium for over 120 days, the adventitious shoots and somatic embryos began to develop into plantlets (Figure 4.5A and 4.5B). By this time, some of the white callus turned green and formed adventitious roots only (Figure 4.5C). There was also increased production of creamy, friable non-regenerable callus with vacuolated cells, which overgrew the cultures.

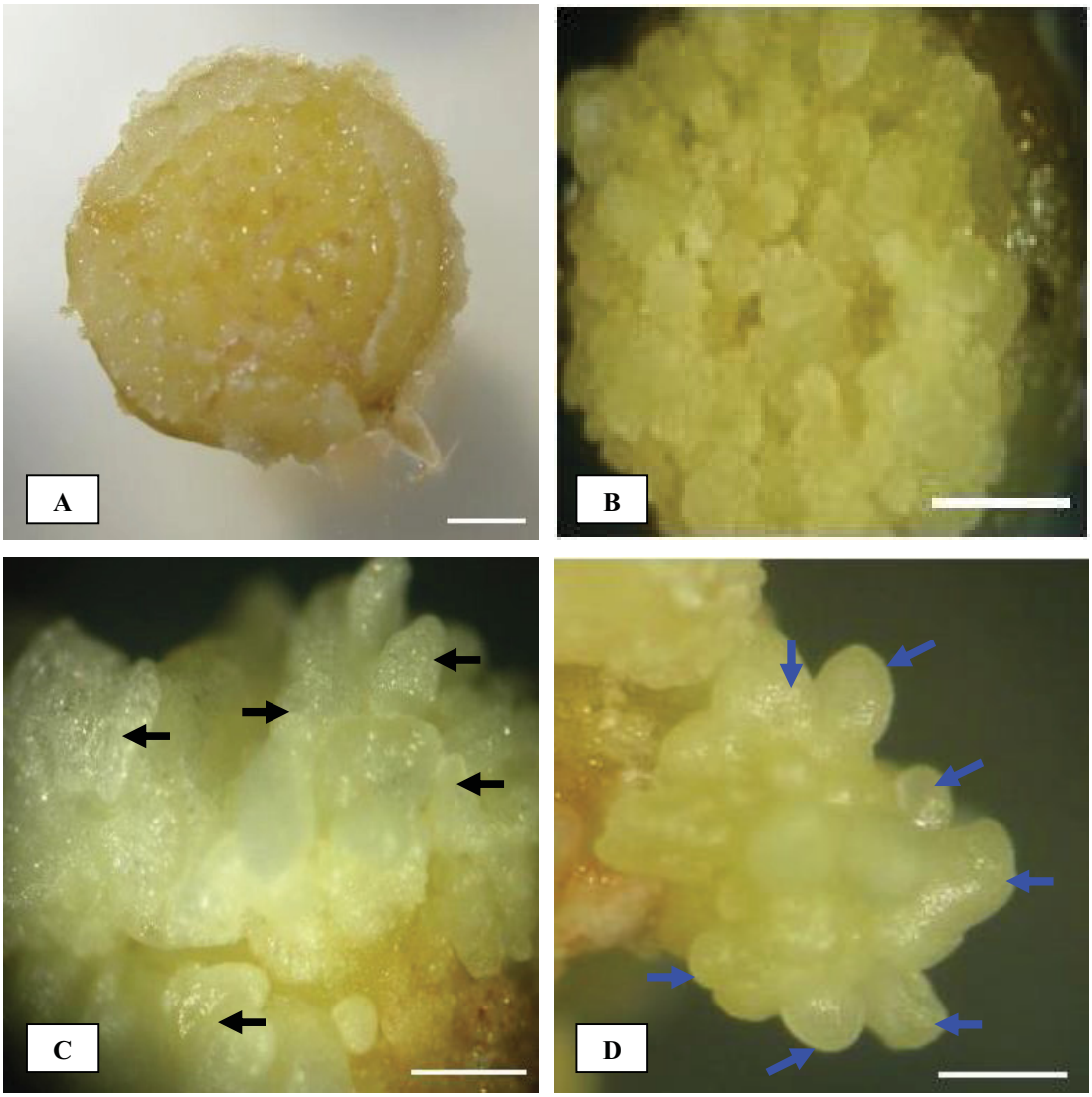


**Figure 4.2:** Taro callus initiation and regeneration from corm explants cv. THA-07. Callus on CIM-I was initially soft and watery (A) but after transfer to CIM-II, it became friable and cream in colour (B). After 45 days on CIM-II what appeared to be adventitious shoots (black arrows) formed on compact white (organogenic) callus (C) and after 75 days what appeared to be somatic embryos (blue arrows) formed on compact, yellow (embryogenic) callus (D). Scale bar = 2 mm (A); 2 mm (B); 3 mm (C); 2 mm (D).

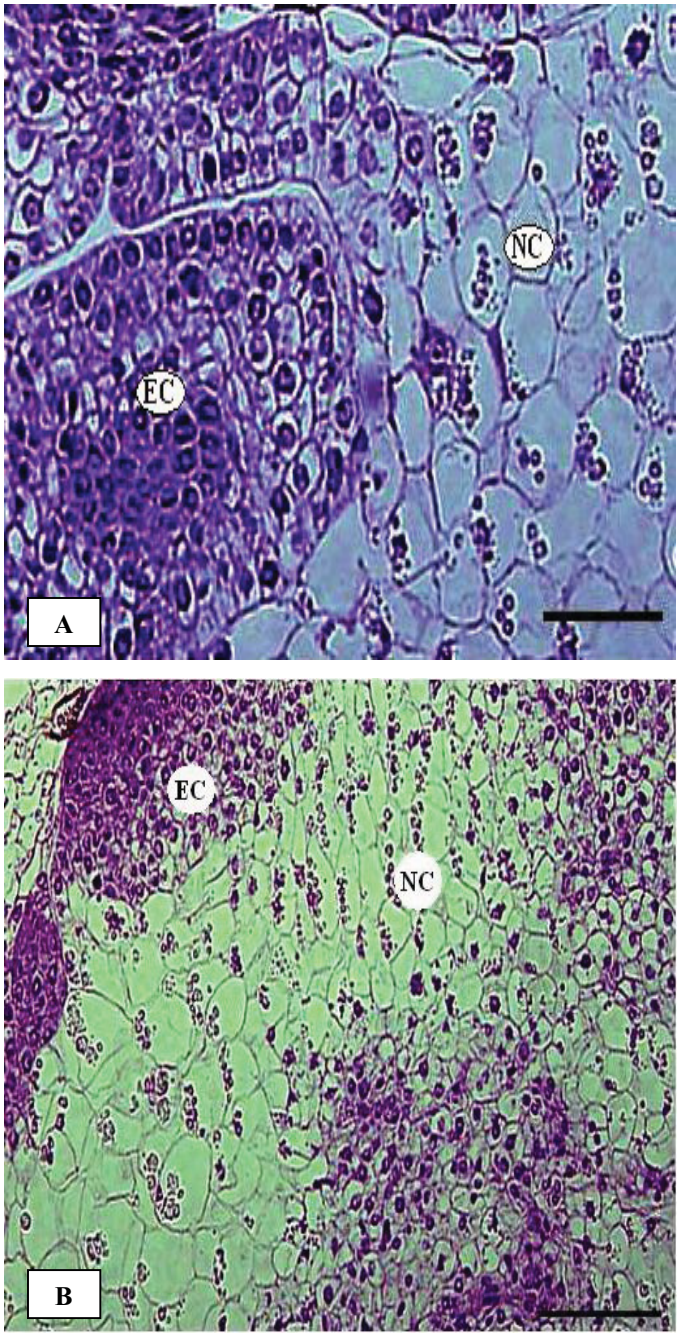


**Figure 4.3:** Different cell types in embryogenic callus in taro cv. CPUK. Embryogenic cells (EC) are small isodiametric cells with a large nucleus and dense cytoplasm while non-embryogenic cells (NC) are large vacuolated parenchymal cells containing plastids (A). The embryogenic cells are in clusters, which are separated by large parenchymal cells (B). Scale bar = 50 μm (A); 200 μm (B).

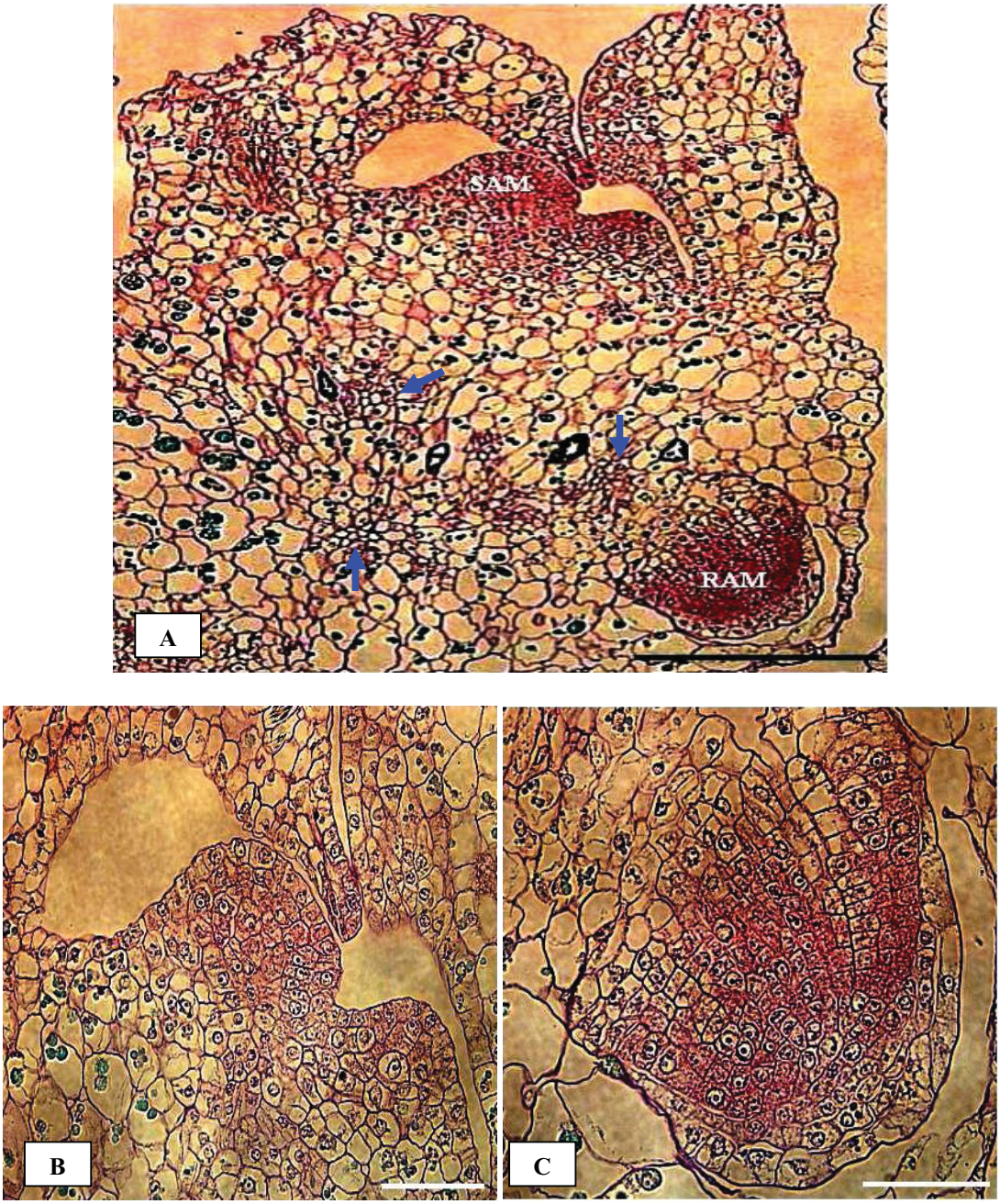


**Figure 4.4:** Histology of taro somatic embryos. (A) Mature somatic embryo showing shoot meristem (SAM) and root meristem (RAM) with vascular connections (blue arrows). Close up view of SAM (B) and RAM (C). Scale bar = 200 µm (A), 50 µm (B and C)

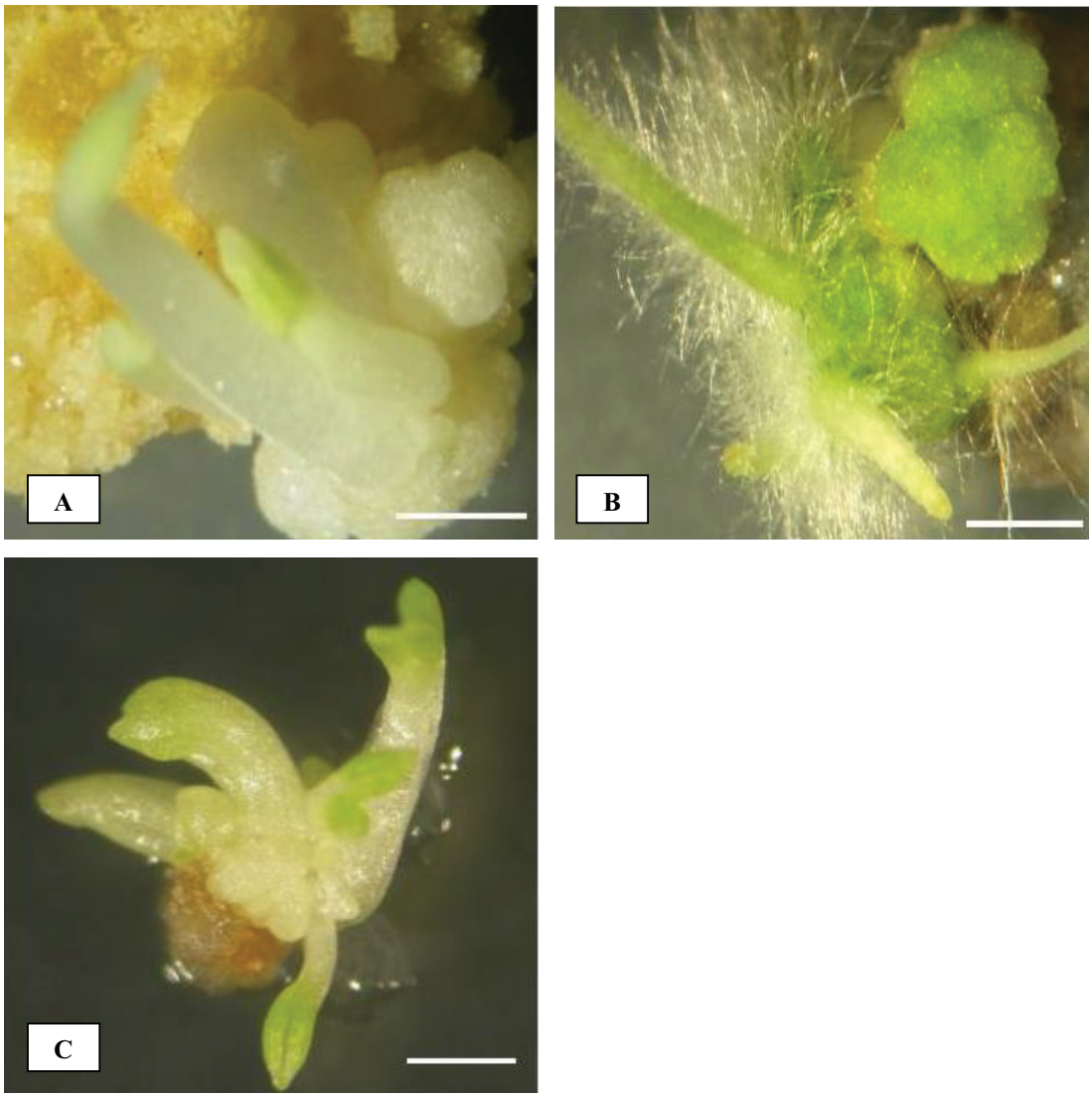


**Figure 4.5:** Taro plantlet regeneration via adventitious shoots (A) and adventitious roots (B) on white callus and somatic embryogenesis on yellow nodular callus (C) cv. CPUK. Scale bar = 4 mm (A), 2 mm (B) and (C).

The percentage of explants producing embryogenic callus ranged from 2.4 % to 34.8 % with the highest percentage observed from treatment with 2.0 mg/L 2,4-D followed by 1.0 mg/L TDZ (Table 4.2). In contrast, the percentage of explants producing organogenic callus ranged from 28.4 % to 67.8 % with the highest percentage being from treatment with 1.0 mg/L 2,4-D followed by 2.0 mg/L TDZ.

To further investigate the conditions optimal for embryogenesis, a second experiment was conducted in which the 2,4-D concentration was fixed at 1.0 mg/L or 2.0 mg/L while TDZ concentration ranged from 0.25 mg/L to 2.0 mg/L (Table 4.3). From all the possible combinations, 2.0 mg/L (2,4-D) followed by 1.0 mg/L (TDZ) again resulted in the highest percentage (31.8 %) of explants producing embryogenic callus. This frequency was significantly higher than all other treatments except 1.0 mg/L 2,4-D and 0.5 mg/L TDZ (27.1 %). The highest frequency of organogenesis occurred where 1.0 mg/L 2,4-D and 0.5 mg/L TDZ (64.2 %) was used; however, the differences between treatments were not as great. Further, organogenic frequency was less consistent between the two experiments represented in Tables 4.2 and 4.3. For example, in the first experiment, the use of 2.0 mg/L TDZ following 1.0 mg/L 2,4-D resulted in the greatest frequency of organogenic callus formation than using 1.0 mg/L TDZ whereas in the second experiment the frequencies were reversed.

### 4.3.3 Duration of exposure of explants to 2,4-D

To further investigate the regenerative response, explants were incubated on 2,4-D for different time periods before transfer to TDZ (Table 4.4). Exposure for duration of 10 or 40 days resulted in significantly fewer explants producing embryogenic callus than for 20 days (3.1 %, 11.9 % and 30.8 %, respectively). Again, the difference in the frequency of the organogenic response was not as great between treatments.

**Table 4.2:** Effect of 2,4-D and TDZ on callus initiation and regeneration in taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Corm explants were maintained on various concentrations of 2,4-D for 20 days followed by transfer to various concentrations of TDZ.

| Plant Growth Regulator (mg/L) | | Total number of explants inoculated | Mean % of total explants producing callus | Mean % of explants producing embryogenic callus | Mean number of embryogenic calli per explant | Mean % of explants producing organogenic callus | Mean number of organogenic calli per explant |
|---|---|---|---|---|---|---|---|
| 2,4-D | TDZ | | | | | | |
| | 0 | 100 | 0 | 0 | 0 | 0 | 0 |
| 0 | 1 | 100 | 85.8 ± 4.7 | 0 | 0 | 0 | 0 |
| | 2 | 100 | 97.0 ± 2.1 | 0 | 0 | 0 | 0 |
| | 0 | 83 | 78.9 ± 6.5 | 0 | 0 | 0 | 0 |
| 1 | 1 | 91 | 94.6 ± 4.1 | 4 ± 3.1 [a] | 0.4 ± 0.31 | 45.1± 3.9 [a] | 2.1 ± 0.76 |
| | 2 | 93 | 99.0 ± 1.0 | 8.8 ± 2.8 [a] | 0.7 ± 0.2 | 67.8 ± 5.1 [b] | 2.6 ±0.32 |
| | 0 | 100 | 96.9 ± 1.6 | 0 | 0 | 0 | 0 |
| 2 | 1 | 100 | 98.0 ± 2.0 | 34.8 ± 2.2 [b] | 1.4± 0.08 | 63.3 ± 6.0 [ab] | 2.4 ± 0.2 |
| | 2 | 110 | 98.2 ± 1.2 | 7.3 ± 2.6 [a] | 0.5 ± 0.17 | 62.8 ± 3.5 [ab] | 2.4 ± 0.11 |
| | 0 | 96 | 65.5 ± 10.0 | 0 | 0 | 0 | 0 |
| 4 | 1 | 90 | 82.0 ± 6.0 | 2.4 ± 1.6 [a] | 0.2 ± 0.13 | 28.4 ± 5.5 [ac] | 1.4 ± 0.24 |
| | 2 | 92 | 95.0 ± 3.0 | 3.5 ± 1.8 [a] | 0.3 ± 0.15 | 34.0 ± 5.2 [ac] | 1.5 ± 0.2 |

Values with means ± SEM are derived from 10 replicate Petri dishes with 8-11 explants per replicate. Means followed by the same letters were not significantly different ($p<0.05$).

**Table 4.3:** Further refinement of 2,4-D and TDZ concentrations required for callus initiation and regeneration in taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Corm explants were maintained on various concentrations of 2,4-D for 20 days followed by transfer to various concentrations of TDZ.

| Plant Growth Regulator (mg/L) | | Total number of explants inoculated | Mean % of total explants producing callus | Mean % of explants producing embryogenic callus | Mean number of embryogenic calli per explant | Mean % of explants producing organogenic callus | Mean number of organogenic calli per explant |
|---|---|---|---|---|---|---|---|
| 2,4-D | TDZ | | | | | | |
| 1 | 0.25 | 108 | 97 ± 3.0 | 17.5 ± 3.1 [a] | 1.1 ± 0.16 | 61.3 ± 5.0 [a] | 2.0 ± 0.18 |
| | 0.5 | 120 | 100 ± 0 | 27.1 ± 4.9 [b] | 1.2 ± 0.15 | 64.2 ± 4.5 [a] | 1.5 ± 0.15 |
| | 1 | 130 | 96.2 ± 2.6 | 13.5 ± 3.4 [a] | 0.73 ± 0.16 | 56.9 ± 5.7 [ac] | 1.7 ± 0.11 |
| | 2 | 115 | 96.4 ± 1.9 | 10.3 ± 3.4 [ac] | 0.73 ± 0.16 | 38.5 ± 4.9 [b] | 1.5 ± 0.11 |
| 2 | 0.25 | 110 | 100 ± 0 | 9.7 ± 2.3 [ac] | 0.7 ± 0.15 | 55.3 ± 5.7 [ab] | 2.0 ± 0.06 |
| | 0.5 | 110 | 88.2 ± 5.6 | 2.2 ± 1.5 [c] | 0.2 ± 0.13 | 50.3 ± 9.2 [ab] | 1.6 ± 0.22 |
| | 1 | 100 | 100 ± 0 | 31.8 ± 2.4 [b] | 1.1 ± 0.06 | 52.9 ± 7.6 [ab] | 1.7 ± 0.09 |
| | 2 | 100 | 95.5 ± 2.4 | 3.7 ± 2.5 [c] | 0.2 ± 0.13 | 42.7 ± 6.8 [bc] | 1.3 ± 0.20 |

Values with means ± SEM are derived from 10 replicate Petri dishes with 10-13 explants per replicate. Means followed by the same letters were not significantly different ($p<0.05$)

**Table 4.4:** The effect of 2,4-D exposure period on callus initiation and regeneration in corm explants of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Explants were exposed to 2,4-D (2.0 mg/L) followed by transfer to TDZ (1.0 mg/L).

| Duration of exposure to 2,4-D (Days) | Total number of explants inoculated | Mean % of total explants producing callus | Mean % of explants producing embryogenic callus | Mean number of embryogenic calli per explant | Mean % of explants producing organogenic callus | Mean number of organogenic calli per explant |
|---|---|---|---|---|---|---|
| 10 | 100 | 99 ± 1.0 | 3.1 ± 1.6 [a] | 0.3 ± 0.15 | 32.6 ± 7.4 [a] | 1.3 ± 0.20 |
| 20 | 130 | 99.2 ± 0.8 | 30.8 ± 4.3 [b] | 1.2 ± 0.07 | 63.8 ± 3.6 [b] | 2.0 ± 0.14 |
| 40 | 160 | 100 ± 0 | 11.9 ± 2.5 [c] | 1.0 ± 0.19 | 51.9 ± 5.2 [b] | 1.9 ± 0.12 |

Values with means ± SEM are derived from 10 replicate Petri dishes with 10 – 16 explants per replicate. Means followed by the same letters were not significantly different ($p<0.05$).

### 4.3.4 Effect of concentration of salts and vitamins in basal medium

The effect of inorganic salts and vitamins on the formation of regenerable callus was also investigated (Table 4.5). Explants were cultured on media containing 2.0 mg/L 2,4-D for 20 days followed by transfer to media containing 1.0 mg/L TDZ. For both mediums, the salts and vitamin concentrations were either full, half or quarter-strength MS. At these concentrations, the percentage of explants producing embryogenic callus was 5.2 %, 32.9 % and 8.7 %, respectively, while for explants producing organogenic callus, the respective response rate was 26.8 %, 73,6 % and 46.6 %. From the above experiments it was determined that the optimal medium for initiation of regenerable callus was half-strength MS medium with a two-step culture firstly on 2.0 mg/L 2,4-D for 20 days followed by transfer to 1.0 mg/L TDZ.

### 4.3.5 Effect of plant genotype on embryogenic callus

The effect of genotype was examined by comparing the response of three esculenta varieties, CK-07, CPUK and THA-07, on two hormone regimes (Table 4.6). There was no significant difference between the genotypes response on the frequency of embryogenic callus formation when treated with 1.0 mg/L 2,4-D and 0.5 mg/L TDZ; 22 % for THA-07 and approximately 30 % for both CK-07 and CPUK. However, when treated with 2,4-D (2.0 mg/L) and TDZ (1.0 mg/L), genotype differences became obvious. The frequency of the explants undergoing embryogenesis in THA-07 was significantly lower (13 %) when compared with CK-07 (30 %) and CPUK (29 %).

A similar pattern was also observed in the organogenic response with no significant difference on 1.0/0.5 (2,4-D/TDZ) combination (approximately 65 % in all cultivars). In contrast, a significant difference in organogenic response was observed on 2.0/1.0 (2,4-D/TDZ) combinations; 59 % for THA-07 compared to approximately 76 % in both CK-07 and CPUK.

**Table 4.5:** The effect of MS medium concentrations on callus initiation and regeneration in corm explants of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Corm explants were maintained on medium containing 2.0 mg/L 2,4-D for 20 days followed by transfer to medium containing 1.0 mg/L TDZ.

| Concentration of MS salts and vitamins | Total number of explants inoculated | Mean % of total explants producing callus | Mean % of explants producing embryogenic callus | Mean number of embryogenic calli per explant | Mean % of explants producing organogenic callus | Mean number of organogenic calli per explant |
|---|---|---|---|---|---|---|
| ¼ MS | 140 | 100 ± 0 | 8.7 ± 2.8 [a] | 0.7 ± 0.15 | 46.6 ± 4.7 [a] | 1.3 ± 0.08 |
| ½ MS | 140 | 100 ± 0 | 32.9 ± 3.1 [b] | 1.2 ± 0.10 | 73.6 ± 2.2 [b] | 2.7 ± 0.22 |
| 1 MS | 140 | 95.7 ± 1.6 | 5.2 ± 1.9 [a] | 0.5 ± 0.17 | 26.8 ± 4.2 [c] | 1.2 ± 0.08 |

Values with means ± SEM are derived from 10 replicate Petri dishes with 14 explants per replicate. Means followed by the same letters were not significantly different ($p<0.05$).

**Table 4.6:** The effect of genotype on callus initiation and regeneration in taro (*Colocasia esculenta* var. *esculenta*). Corm explants were maintained on medium containing 2,4-D (2.0 mg/L and 1.0 mg/L) for 20 days followed by transfer to medium containing TDZ (1.0 mg/L and 0.5 mg/L, respectively).

| Cultivar | Hormone regime (2,4-D / TDZ) (mg/L) | Total number of explants inoculated | Mean % of total explants producing callus | Mean % of explants producing embryogenic callus | Mean number of embryogenic calli per explant | Mean % of explants producing organogenic callus | Mean number of organogenic calli per explant |
|---|---|---|---|---|---|---|---|
| THA-07 | 1.0 / 0.5 | 160 | 100 | $21.9 \pm 4.4^{a}$ | $1.2 \pm 0.86$ | $67.3 \pm 3.9^{b}$ | $1.9 \pm 0.17$ |
| | 2.0 / 1.0 | 160 | 100 | $13.2 \pm 3.8^{a}$ | $1.0 \pm 0.16$ | $59.4 \pm 3.4^{a}$ | $1.7 \pm 0.15$ |
| CK-07 | 1.0 / 0.5 | 140 | 97.9 | $31.9 \pm 5.7^{a}$ | $1.4 \pm 0.21$ | $66.7 \pm 6.3^{b}$ | $3.0 \pm 0.30$ |
| | 2.0 / 1.0 | 140 | 94.3 | $30.0 \pm 3.8^{b}$ | $1.5 \pm 0.10$ | $77.9 \pm 3.9^{b}$ | $2.4 \pm 0.19$ |
| CPUK | 1.0 / 0.5 | 150 | 100 | $18.7 \pm 3.4^{a}$ | $1.1 \pm 0.14$ | $60.7 \pm 7.0^{b}$ | $2.1 \pm 0.23$ |
| | 2.0 / 1.0 | 140 | 100 | $28.6 \pm 2.6^{b}$ | $1.2 \pm 0.07$ | $73.6 \pm 2.2^{b}$ | $2.7 \pm 0.22$ |

Values with means ± SEM are derived from 10 replicate Petri dishes with 14 –16 explants per replicate. Means followed by the same letters were not significantly different ($p<0.05$).

## *4.4 Discussion*

In this study, a two step-protocol was developed to initiate embryogenic callus from corm explants in taro. Initially, the explants were cultured on half-strength MS medium containing 2.0 mg/L 2,4-D (CIM-I) and maintained in darkness for 20 days at 25 $^{o}$C followed by transfer of the explants onto half-strength MS containing 1.0 mg/L TDZ (CIM-II) while maintaining the same light and temperature conditions.

The events leading to the formation of embryogenic callus occurred in succession. Initially, soft watery callus was formed, followed by white-cream friable callus both of which were non-regenerable. This was followed by the formation of white callus, which was glossy and compact with some early shoot development present. Finally, pale yellow–cream coloured nodular callus appeared, which was glossy and compact with translucent globular structures present. The morphology of white compact and yellow nodular calluses were similar to the Type-I and Type-II callus in maize, respectively (Vasil *et al.*, 1984; Jiménez and Bangerth, 2001b). The yellow, nodular callus has also been reported as embryogenic in date palm (Huong *et al.*, 1999) and sugar cane (Gandonou *et al.*, 2005). The sequential formation of different types of callus observed in the current study could be the result of changing conditions within culture medium over time so that at different stages, the existing conditions favoured the formation of different callus types.

Somatic embryos are characteristically translucent globular structures with a distinct epidermis (Vasil *et al.*, 1984). As a further confirmation, histological studies of callus and putative somatic embryos were undertaken. The embryogenic callus contained two distinct cell types: i) embryogenic cells which were small, isodiametric and had large nucleus and a dense cytoplasm and ii) non-embryogenic which were large vacuolated cells with few plastids. The embryogenic cells were in clusters located generally on the periphery of the callus mass, interspersed with non-embryogenic parenchymal cells. Similar embryogenic cell clusters have been reported to form the 'pre-embryogenic units' (globular embryos) in maize (Šamaj *et al.*, 2003). The co-existence of these cell types shows that only a small proportion of embryogenic cells

are present in the callus and the role of large parenchymal cells may be as 'nurse cells' (Ogita *et al.*, 2001). When histological sections of mature somatic embryos were observed, they contained two meristems. These were most likely root and shoot poles, an indicator that these structures are true somatic embryos (Thompson *et al.*, 2001).

The frequency of embryogenesis varied with the concentration of 2,4-D in the induction medium. The concentration of 2,4-D had a significant effect on the percentage of taro corm explants forming embryogenic callus, with a lower percentage of explants forming embryogenic calluses using low (1.0 mg/L) and high (4.0 mg/L) concentrations of 2,4-D but a high frequency using 2.0 mg/L. A similar result was also observed in the graminacious monocot, bahiagrass "Nanou"(Chen *et al.*, 2001). Incorporation of very high levels of 2,4-D (10-50 mg/L) also proved ineffective (data not shown) as all the explants turned necrotic between one to two months after exposure. Similarly, very low concentrations of 2,4-D (0.25 mg/L, 0.5 mg/L) supported some callus formation but the concentration may have been too low to make the cells competent for embryogenesis. It should be noted that high concentrations of this growth regulator have been reported to cause somaclonal variation in some species (Fernando *et al.*, 2002). In contrast, no somaclonal variation was reported in guinea grass (*Panicum maximum* jacq) despite using 10 mg/L 2,4-D for embryogenesis (Lu and Vasil, 1982). This demonstrates that different species of plants have different requirements for 2,4-D but as a precaution, the minimum effective concentration should be used to avoid somaclonal variation.

There is evidence that 2,4-D induces somatic embryogenesis by influencing endogenous IAA in explant tissues. For example, embryogenic carrot cells grown in the presence of 2,4-D contained high levels of IAA (Ribnicky *et al.*, 1996). High levels of endogenous IAA were also found in embryogenic callus cultures of maize and carrot and a loss in embryogenic competence of the calli due to prolonged time of culture occurred concomitantly with a reduction in the IAA levels. Thus, a high IAA concentration may be required to induce the competence of cells to undergo embryogenesis (Jiménez and Bangerth, 2001a; Jiménez and Bangerth, 2001b).

Consequently, it is possible that 2,4-D influenced somatic embryogenesis in *Colocasia esculenta* var. *esculenta* indirectly by modulating the endogenous IAA levels in corm explant tissue. Recently it has been proposed that 2,4-D may initiate somatic embryogenesis by inducing a stress response in plant cells (Pasternak *et al.*, 2002; Shinoyama *et al.*, 2004). Expression of stress related genes has been found in the early stages of embryogenesis, thus it has been proposed that this is an extreme stress response in cultured plant cells (Pasternak *et al.*, 2002). It may also be that embryogenesis in taro is stress related and not entirely due to PGRs. The prolonged incubation of the explants (four months) without subculture may place the explants under stress since, nutrition depletion, accumulation of toxics compounds and water stress would occur after such a long incubation time.

Similarly, TDZ also had a significant effect on the percentage of corm explants forming embryogenic callus; high (2.0 mg/L) and low (0.5, 1.0 mg/L) concentrations exhibited inhibitory and promoting effects, respectively. Low concentrations of TDZ (0.5-1.0 mg/L) have been reported to promote callus induction, somatic embryo formation and germination in other monocots such as *Colocasia esculenta* var. *antiquorum* (Thinh, 1997), banana (Srangsam and Kanchanapoom, 2003) and bamboo (Lin *et al.*, 2004).

The mode of action of TDZ is currently unclear. It has been hypothesized that TDZ either directly promotes somatic embryogenesis due to its own biological activity (Visser *et al.*, 1992), or it affects the endogenous ratios of endogenous auxins and cytokinins (Visser *et al.*, 1992; Panaia *et al.*, 2004; Thomas and Puthur, 2004). TDZ has been shown to induce the accumulation of both endogenous auxins and cytokinins in legumes and herbaceous plants (Bhuiyan and Adachi, 2002). Since taro is believed to contain high levels of endogenous cytokinins (Taylor, pers. comm.), which are inhibitory to somatic embryogenesis (Wenck *et al.*, 1988), and as high levels of endogenous IAA are correlated with somatic embryogenesis (Ribnicky *et al.*, 1996; Jiménez and Bangerth, 2001a; Jiménez and Bangerth, 2001b), it is possible that TDZ induces somatic embryogenesis in *C. esculenta* var. *esculenta* by modulating endogenous auxin/cytokinin levels, in particular by effecting IAA gradients within the

explant. The late initiation of embryogenic callus in corm explants of taro also indicates that the establishment of optimum auxin/cytokinin balance is a slow and gradual process in taro.

Interestingly, 2,4-D and TDZ did not induce regenerable callus independently in this study and were only effective when used in succession. In contrast, regenerable callus (organogenic only) has been initiated using either 2,4-D or TDZ as the sole growth regulator in a small number of related monocot aroids; in *C. esculenta* var. *antiquorum* from etiolated stem explants using 2.0 mg/L 2,4-D (Murakami *et al.*, 1995) and in cocoyam (*Xanthosoma sagittifolium*) shoot-tip and petiole explants using 0.01mg/L TDZ (Nyochembeng and Garton, 1998). Further, culturing petiole explants from the fourth leaf primordia on 1.0 mg/L TDZ resulted in direct somatic embryogenesis in *C. esculenta* var. *antiquorum* (Thinh, 1997). It would appear that explant tissues of *C. esculenta* var. *esculenta* are not as easily manipulated as *antiquorum* as they required the collaborative effect of two growth regulators. It is possible that 2,4-D was required to make the cells competent for embryogenesis/organogenesis, and the subsequent transfer to CIM-II TDZ was the trigger for the formation of regenerable callus. The late initiation of embryogenic callus could also be due to the decreasing concentrations of 2,4-D and TDZ and other factors in the initiation medium such as nutrient depletion and water stress, which upon reaching a critical concentration, triggered embryogenesis. This also demonstrated the recalcitrant nature of *Colocasia esculenta* var. *esculenta,* since it required two very potent growth regulators to form embryogenic callus. Verma *et al* (2004) reported direct somatic embryogenesis in taro by culturing petiole explants on 2,4-D (2.0 mg/L) and TDZ (0.4 mg/L) for four weeks followed by transfer onto TDZ (1.0 mg/L). Similarly, Gairi and Rashid (2004) also initiated somatic embryogenesis in a "non-responsive" rice cultivar by exposing the explants to 2,4-D (4.4 mg/L) for three days followed by transfer onto TDZ (2.2 mg/L). These reports show that exposure to 2,4-D followed by transfer to TDZ at half the concentration of 2,4-D used initially can be used to promote somatic embryogenesis in some monocotyledonous species. However, other two-step protocols have been used in other species. For example, somatic embryos of quince (*Cydonia oblonga* Mill) were initiated by

subjecting leaf explants to 2,4-D for two days followed by transfer onto medium enriched with both kinetin and NAA (D'Onofrio and Morini, 2006). In this case exposure to 2,4-D was required to make the cells competent for embryogenesis but transfer to other PGR(s) was required to trigger these competent cells to undergo dedifferentiation, and hence produce embryogenic callus and subsequently somatic embryos.

The duration of exposure of the explants to 2,4-D was also critical in taro somatic embryogenesis, since exposure for 10 days and 40 days did not favour embryogenesis whereas exposure for 20 days significantly increased the percentage of explants producing embryogenic callus. It would appear that explants require a threshold of auxin concentration and exposure time to attain embryogenic competence but that an upper limit of concentration or exposure could be exceeded. Prolonged exposure to 2,4-D has also been reported to decrease somatic embryogenesis in rice (Gairi and Rashid, 2004). The requirements for specific growth regulator(s) may also depend on the morphological and biochemical nature of the particular explants (Dhar and Joshi, 2005). For example, in carrot, petiole explants, hypocotyl explants and single cells isolated from established suspension cultures required exposure to exogenous auxin for one, two or seven days, respectively, before they were competent to undergo embryogenesis (Zimmerman, 1993). Thus, for taro (*Colocasia esculenta* var. *esculenta*) corm explants, twenty days exposure to 2.0 mg/L 2,4-D followed by subculture onto 1.0 mg/L TDZ was optimal for the initiation of embryogenic callus.

Various explants including petioles, shoot-tip, meristems, leaf primordia and axillary buds have been utilized in taro tissue culture (Abo El-Nil and Zettler, 1976; Jackson *et al.*, 1977; Yam *et al.*, 1990; Yam *et al.*, 1991; Chng and Goh, 1994; Verma *et al.*, 2004). Chng and Goh (1994) used corm slices to generate shoots for micropropagation but these have not been used for callus induction. In the present study, both embryogenic and organogenic callus was initiated from corm explants. Further, it was shown that corm explants are preferable to petiole, leaf primordial, shoot-tip and axillary bud explants with respect to the type of callus initiated, and the time required for the preparation of explants for *in vitro* culture. In addition, a

substantial quantity of explants can be obtained from one plantlet thus providing the large number of explants required for a significant number of experimental replicates. Furthermore, corm explants did not require the addition of complex organic additives such as taro corm extract and coconut water, which were reported as essential requirements for callus initiation from shoot-tip and axillary buds (Yam *et al.*, 1990).

Attempts to initiate embryogenic or organogenic callus from mid-rib, root-tip, third and fourth leaf primordia and shoot-tips were unsuccessful (data not shown). However, some petioles from third leaf primordia and shoot-tips did form non-embryogenic callus. Since axillary buds are present in taro corms, such an explant would contain regions of meristematic cells and these may be contributing to the formation of regenerable callus. Meristematic tissues have proved to be the most suitable explant for somatic embryogenesis (Lakshmanan and Taji, 2000). Generally, in adequate culture conditions, the meristematic tissues/cells are competent to embark on an embryogenesis developmental program thus resulting in the production of somatic embryos (Guerra *et al.*, 2001).

There was a marked difference in the frequency of callus formation from different regions of the corm. The explants from the top region formed most callus (including embryogenic and organogenic) followed by the mid region, with the basal part forming the least amount of callus (observation made in this study). A similar response was also seen with petiole explants even though the callus was non-embryogenic. Variation in the frequency of callus formation between the different regions of the same explants was also observed in petiole explants of celery (Williams and Collins, 1976) and in hypocotyl explants of eggplant (Sharma and Rajam, 1995). This differing response according to the region of the explant could be due to the developmental stage of the cells, varying levels of endogenous growth regulators or the distribution of meristematic cells.

The concentration of inorganic salts also had an effect on regeneration, with a 5 to 8-fold increase in the formation of embryogenic callus on half-strength MS compared with quarter and full-strength MS medium. This may be a nutritional, osmotic or ion

concentration requirement. Yam *et al* (1990; 1991) also reported callus initiation from axillary buds of *C. esculenta* var. *esculenta* on half-strength MS medium. In contrast, full-strength MS medium was used for direct somatic embryogenesis from petiole explants in *C. esculenta* var. *antiquorum* (Thinh, 1997). Thus, this finding is in agreement with the views of Yam *et al* (1991) that explant tissues of *C. esculenta* var. *esculenta* require dilute media.

The three cultivars of taro had variable responses to somatic embryogenesis and such genotypic variation has been observed in other species (Kim, Park *et al.*, 2004; Kayim and Koc, 2006). In cotton, only a limited number of varieties could be induced to produce embryogenic callus (Zhang *et al.*, 2001). Consequently, for *Colocasia esculenta* var. *esculenta* it may be that genotype has some moderate effect although more cultivars need to be examined to determine the full extent of genotypic effects. The fact that the three taro cultivars examined did not differ significantly in their response to embryogenesis when treated with 1.0 mg/L 2,4-D and 0.5 mg/L TDZ but did when treated with 2.0 mg/L 2,4-D and 1.0 mg/L TDZ demonstrated some genotype effect.

Preliminary experiments showed that light inhibited callus initiation with no callus formation from explants incubated in light. Similarly, dark culture promoted the formation of embryogenic callus in maize (McCain *et al.*, 1988), wheat (Mahalakshmi *et al.*, 2003) and barley (Sharma *et al.*, 2005). In the present study, approximately 50 % of corm explants released traces of brown and white exudates. Thus, the inability to initiate callus in light may be due to the production of phenolic compounds which are induced by light and inhibitory to callus formation (Evans *et al.*, 1983). However, callus in taro has been initiated both in the light (Jackson *et al.*, 1977; Yam *et al.*, 1990; Yam *et al.*, 1991) and dark (Abo El-Nil and Zettler, 1976) with no previous reports of phenolic compound production being problematic during light culture. Therefore, it is possible that light has other influences such as promoting differentiation (Slater *et al.*, 2003).

In summary, by modifying and optimizing various *in vitro* parameters, a highly efficient and reproducible protocol for the initiation of embryogenic callus was established for *Colocasia esculenta* var. *esculenta* which was applicable to the three cultivars examined. This is significant as previous reports for regeneration in the *esculenta* variety have been difficult to repeat and a reliable regeneration system is the first requirement for any molecular breeding program to be successful. Also of importance is a method for proliferating regenerable callus as this provides sufficient target tissue for transformation and efficient recovery following transformation.

# CHAPTER 5

## Factors Affecting Callus Proliferation and Regeneration in Taro (*Colocasia esculenta* var. *esculenta*)

### *5.1 Introduction*

Callus proliferation is one means of multiplying and maintaining a continuous supply of regenerable plant material. This is particularly useful in those species for which the formation of embryogenic callus is a lengthy process. Such proliferation has been achieved in liquid media for species such as carrot (Zimmerman, 1993), banana (Becker, 1999), ginger (Guo and Zhang, 2005), potato (Vargas *et al.*, 2005) and bitter melon (Thiruvengadam *et al.*, 2006), and on solid media for species such as maize (Claparols *et al.*, 1993), egg plant (Yadav and Rajam, 1998), sorghum (Mishra and Khurana, 2003) and sugarcane (Gandonou *et al.*, 2005).

Irrespective of the mode of proliferation, callus maintenance medium (CMM) has a profound effect on regeneration efficiency. An effective CMM should maintain good cell proliferation without significantly reducing the regeneration capacity thus allowing the generation of large numbers of plantlets or the development of an efficient transformation system. For example, in carrot suspension cells, modified LS medium containing zeatin, 2,4-D and $CaCl_2$ (15 mM) significantly increased the proportion of single cells in suspension culture capable of forming somatic embryos up to 85-90 % (Nomura and Komamine, 1985).

The CMM developed for a particular species is usually a modification of the callus initiation medium (CIM) although sometimes they are identical. In sweet potato, for example, MS medium supplemented with 2,4-D (2.0 mg/L) was used for both callus initiation and also for the initiation and maintenance of cell suspension cultures (Liu *et al.*, 1998). In taro, proliferation of organogenic callus derived from axillary buds

was achieved on solid medium using half-strength MS containing NAA, BAP and taro corm extract (Yam *et al.*, 1990) or half-strength MS salts containing 2,4,5-T and taro corm extract (Yam *et al.*, 1991). The former medium was used for callus proliferation only while the latter was also used for callus initiation.

There are no reported methods for the generation of taro cell suspension cultures. The development of such methods would not only enable the rapid generation of large amounts of regenerable material but would also enable the synchronization of somatic embryogenesis thus facilitating the production of embryos and plants of uniform age and size for planting (Attree *et al.*, 1995). The objectives of the research contained in this chapter were to (i) develop an effective CMM for the proliferation of taro embryogenic callus, (ii) establish taro embryogenic cell suspension cultures and (iii) develop an effective medium for the initiation, maturation and germination of taro somatic embryos.

## *5.2 Materials and Methods*

### 5.2.1 Callus proliferation on solid medium

Culture medium for callus growth and proliferation was the same as for CIM (Chapter 4) except that hormone concentrations were altered and glutamine added. Media contained either TDZ (0, 0.5, 1.0 mg/L), 2,4-D (0, 0.5, 1.0 mg/L), TDZ and 2,4-D (0.5 + 0.5), (1.0 + 0.5), (1.0 + 1.0), (0.5 + 1.0) or TDZ, 2,4-D (1.0 mg/L + 0.5 mg/L) and glutamine (800, 1600, 2400 mg/L). Filter sterilized (0.2 μm pore size, Cole-Parmer$^{R}$) glutamine was added to medium after autoclaving. Hereafter callus proliferation medium is referred to as callus maintenance medium (CMM).

Callus deemed to be embryogenic by the presence of somatic embryos was cut into equal sizes (2 mm x 2 mm x 1 mm) and placed onto CMM. For each combination of 2,4-D, TDZ and glutamine, seven to ten pieces of callus were placed on each of seven CMM plates. Cultures continued to be maintained in the dark and were checked periodically for callus growth. After two months, the fresh weight of each callus piece

was recorded as an indicator of proliferation. Following the first two months on CMM without subculture, embryogenic callus was maintained by monthly subculture onto fresh CMM.

### 5.2.2 Callus proliferation in liquid medium

#### 5.2.2.1 Initiation of suspension culture

A small amount of embryogenic callus (~0.5 g) was placed into 10 mL of liquid medium in a 100 mL conical flask. This medium contained half-strength MS, TDZ (1.0 mg/L), 2,4-D (0.5 mg/L) and glutamine (100 mg/L) but minus agar. The mouth of the flask was covered with 3-4 layers of sterile aluminium foil to prevent contamination and to minimize evaporation. The entire flask was then covered with aluminium foil and placed on a rotary shaker set at 90 rpm. After 7 days, 10 mL of fresh medium was added. After an additional 7 days, the cells were allowed to settle to the bottom of the flask and 10 mL of the supernatant was removed and replaced with an equal volume of fresh medium. By this time the callus began to dissociate into single cells or form small multicellular clumps. Media was refreshed a second time after which the entire contents of the flask were transferred to 250 mL flasks and the volume was made up to 50 mL. From this point onwards, subculture was at 7-day intervals by either replacing 40 mL of old medium or, if cell density was sufficient, dividing between flasks as described below.

#### 5.2.2.2 Maintenance of suspension culture

Embryogenic suspension cultures were maintained by allowing cells to settle then removing old medium until the remaining volume was 20 mL. Flasks were then agitated to evenly distribute cells and 10 mL transferred to another 250 mL conical flask containing 40 mL of fresh medium. This was done at 2-3 weeks intervals. If the suspension was too dense to simply divide and had large cell clumps, it was passed through a 500 μm stainless steel sieve. From the filtrate, 1 mL settled cell volume (SCV) of suspension cells was dispensed into 50 mL of CMM and maintained as above.

In a separate experiment, the effects of sucrose (20, 30 g/L) and glutamine (100, 400, 800 mg/L) on proliferation and regeneration were examined for further refinement of the liquid medium. Suspension cultures (1 mL SCV) were maintained in liquid medium containing various combinations and concentrations of sucrose and glutamine for one month with weekly subcultures then regeneration potential was determined by plating cells on hormone-free half-strength MS medium.

#### 5.2.2.3 Growth curve

The growth curve of suspension cultures was determined by culturing 1 mL SCV of embryogenic suspension cells in 50 mL of liquid medium in 250 mL conical flasks. Medium consisted of half-strength MS, TDZ (1.0 mg/L), 2,4-D (0.5 mg/L) and glutamine (100 mg/L), and five replicate flasks were used and were treated in the same manner as other cultures mentioned above. The growth rate was measured by transferring the cells into graduated (15 mL) Falcon tubes. The cells were allowed to settle for at least 30 minutes then the volume was recorded. The contents were then transferred back to the flasks and maintained as usual. The growth was monitored daily for a period of 14 days without subculture.

### 5.2.3 Regeneration from embryogenic callus cultured on solid medium

Embryogenic callus was transferred from solid CMM to regeneration medium (RM) and maintained in the dark for two weeks then cultured at low light intensity (5 μmoles photons.$m^{-2}$s). Three media were examined for their effect on somatic embryo initiation, maturation and germination: i) half-strength MS medium containing no PGRs, ii) half-strength MS medium containing 1.0 mg/L ABA and iii) half-strength MS medium containing 0.2 mg/L NAA plus 0.1 mg/L kinetin. The cultures were routinely checked for the presence of somatic embryos. The embryos were allowed to mature and germinate on the same medium. After two months without subculture, data was recorded and the frequency of callus producing somatic embryos and total number of embryos were calculated.

### 5.2.4 Regeneration of somatic embryos from suspension cultures

Four embryogenesis media (EM) were developed and examined for their efficacy in initiating somatic embryos from cell suspensions. The media had the same components as the liquid CMM except the concentrations of growth regulators were altered as follows: $EM_1$= TDZ (1.0 mg/L) + 2,4-D (0.5 mg/L); $EM_2$ = TDZ (0.1 mg/L) + 2,4-D (0.05 mg/L); $EM_3$ = TDZ (0.01 mg/L) + 2,4-D (0.005 mg/L) and $EM_4$ = Zeatin (0.1 mg/L) + NAA (0.05 mg/L).

Suspension cells were collected 4 days after subculture, passed through 500 μm mesh and both the filtrate and cells that did not pass through the filter was collected. The cells were allowed to settle in 50 mL Falcon tubes and sufficient supernatant was removed to leave a settled cell volume/liquid medium ratio of approximately 1:5. The cells were then resuspended and 250 μL aliquots were dispensed directly onto sterile 70 mm Whatman filter paper discs overlaid on various EMs in 90 mm x 15 mm Petri dishes. The Petri dishes were sealed with Parafilm$^{TM}$, wrapped with aluminium foil and maintained at 25 $^{o}$C for two months to allow embryo formation. After determining the optimal EM, embryo formation was further refined by varying sucrose concentration (30, 40 and 50 g/L) and incorporating biotin (1.0 mg/L).

After two months on EM, the pro-embryogenic masses (PEMs) together with the somatic embryos were transferred onto germination media (GM). Two media were examined for their effect on maturation and germination: i) hormone-free half-strength MS (RM) and ii) half-strength MS containing 0.05 mg/L BAP and 0.1 mg/L IAA (GM). The cultures were maintained in darkness for two weeks then incubated under low light intensity.

### 5.2.5 Plant development and recovery

Fully germinated embryos were transferred to half-strength MS medium in individual 28 mL McCartney bottles for further development. After the plantlets reached a height of 4-5 cm, they were acclimatized in a shade house. Plants were potted in a 1:1 mix of soil and perlite. Humidity was maintained by covering the pots with clear

polyethylene bags for one week and watering on alternate days. Data was recorded one month after acclimatization and percentage survival was calculated.

### 5.2.6 Histology of PEMs and somatic embryos

PEMs and somatic embryos were fixed in formaldehyde-glacial acetic acid-ethanol (FAA): 1:1:8 (v/v) for four days. Tissue processing, mounting in wax, sectioning and staining with Safranin-O Fast Green were done as in Chapter 4. Stained sections were viewed using a Leica (Laborlux-S) compound light microscope at 40-1000 times magnification.

## *5.3 Results*

### 5.3.1 Callus growth, proliferation and maintenance on solid medium

To investigate the parameters affecting proliferation, embryogenic callus was removed from the original explants and placed on half-strength MS medium containing various concentrations of 2,4-D and TDZ (Table 5.1). In general, the proliferation of callus increased with increasing concentration of hormones both independently and in combination. This trend continued up to a TDZ and 2,4-D combination of 1.0 mg/L and 0.5 mg/L, respectively. Increasing the concentration of 2,4-D to 1.0 mg/L did not significantly increase callus proliferation, while the effect of TDZ concentrations above 1.0 mg/L was not examined. In general, the presence of 2,4-D had a greater effect than TDZ. This was most obvious in treatments in which no 2,4-D was present where very little or no callus proliferation occurred irrespective of the TDZ concentration. In contrast, callus proliferation occurred in the absence of TDZ. However, the greatest proliferation rate occurred with a combination of 1.0 mg/L TDZ and 0.5 mg/L 2,4-D. This was both in terms of the frequency of callus clumps proliferating (100 %) and the mean fresh weight per callus clump (34.5 mg).

Although a combination of TDZ and 2,4-D (1.0 + 0.5) was effective in inducing callus proliferation, much of the callus became watery and non-regenerable within three to four weeks. To prevent this, the effect of glutamine on embryogenic callus

proliferation was examined (Table 5.2). Glutamine did not have a significant effect on the frequency of callus clumps proliferating. However, when used at a concentration of 800 mg/L, a significant increase in the mean fresh weight per callus clump (52.7 mg) was observed. Further, the callus remained intact (did not become watery) for over a month. Higher glutamine concentrations did not result in a further increase in fresh weight. The above experiments indicated that the optimal medium for callus proliferation on solid medium was half-strength MS containing 1.0 mg/L TDZ, 0.5 mg/L 2,4-D and 800 mg/L glutamine; this medium is henceforth referred to as solid callus maintenance medium ($CMM_S$).

### 5.3.2 Characterization of suspension cultures

Suspension cultures were initiated by transferring approximately 0.5 g embryogenic callus into liquid CMM containing glutamine (100 mg/L) and constantly agitating the medium at 90 rpm in the dark. The inoculated callus increased in size by 2-3 fold in two weeks and began to produce single cells and small cell aggregates by the third week. Initially, most of the cells released into the liquid were large and vacuolated (Figure 5.1A), however, dense cell aggregates began to form with subsequent weekly subcultures (Figure 5.1B).

**Table 5.1:** The effect of TDZ and 2,4-D on embryogenic callus proliferation of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK on agar-solidified medium containing half-strength MS with various concentrations and combinations of TDZ and 2,4-D.

| Plant Growth Regulator (mg/L) | | Total number of callus pieces inoculated | Number of callus pieces undergoing necrosis | Number of callus pieces surviving | Mean % of callus pieces per replicate undergoining proliferation | Mean fresh weight per callus piece (mg) |
|---|---|---|---|---|---|---|
| TDZ | 2,4-D | | | | | |
| 0 | 0 | 49 | 29 | 20 | 0† | 0† |
| | 0.5 | 49 | 22 | 27 | $20.4 \pm 5.3^{b}$ | $16.5 \pm 4.4^{bd}$ |
| | 1 | 49 | 2 | 47 | $20.4 \pm 7.5^{b}$ | $7.9 \pm 2.4^{c}$ |
| 0.5 | 0 | 49 | 26 | 23 | 0† | 0† |
| | 0.5 | 70 | 1 | 69 | $85.7 \pm 6.1^{c}$ | $14.4 \pm 0.96^{bc}$ |
| | 1 | 49 | 1 | 48 | $95.9 \pm 2.6^{cd}$ | $21.7 \pm 2.6^{bd}$ |
| 1 | 0 | 49 | 42 | 7 | $6.1 \pm 6.1^{a}$ | $0.57 \pm 0.57^{a}$ |
| | 0.5 | 49 | 0 | 49 | $100 \pm 0.0^{d}$ | $34.5 \pm 2.6^{e}$ |
| | 1 | 49 | 1 | 48 | $97.9 \pm 2.1^{cd}$ | $23.3 \pm 3.4^{d}$ |

Values with means ± SEM are derived from seven replicate Petri dishes with 7-10 callus explants per replicate.
Means followed by the same letters were not significantly different ($p<0.05$).
†- callus on these treatments turned necrotic, hence weight was not recorded.

**Table 5.2:** The effect of glutamine on embryogenic callus proliferation of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK on agar-solidified medium containing half-strength MS, TDZ (1.0 mg/L) and 2,4-D (0.5 mg/L).

| Concentration of glutamine in medium (mg/L) | Total number of callus pieces inoculated | Number of callus pieces undergoing necrosis | Number of callus pieces surviving | Mean % of callus pieces per replicate undergoing proliferation | Mean fresh weight per callus piece (mg) |
|---|---|---|---|---|---|
| 0 | 49 | 0 | 49 | $100 \pm 0.0^{a}$ | $34.5 \pm 2.6^{a}$ |
| 800 | 52 | 8 | 44 | $88.0 \pm 4.9^{a}$ | $52.7 \pm 10.1^{b}$ |
| 1600 | 46 | 16 | 30 | $64.0 \pm 6.0^{b}$ | $22.4 \pm 6.9^{a}$ |
| 2400 | 50 | 4 | 46 | $92.0 \pm 3.7^{a}$ | $25.0 \pm 4.7^{a}$ |

Values with means ± SEM are derived from of five replicate Petri dishes with 8-12 callus pieces per replicate.
Means followed by the same letters were not significantly different ($p<0.05$).

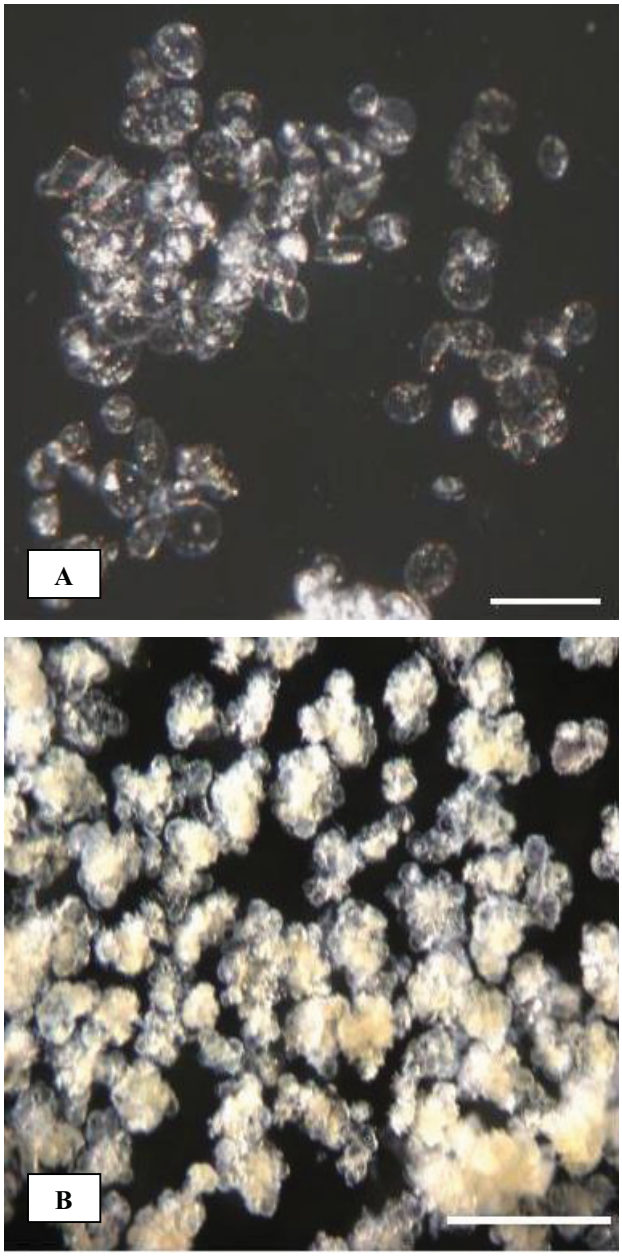


**Figure 5.1:** Types of cell aggregates in suspension cell culture of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Initially callus formed large vacuolated cells (A), but over time yellow, dense, small multicellular aggregates began to form (B). Scale bar = 0.5 mm (A); 1 mm (B).

Suspension cultures contained two distinct cell types: spherical dense cells with small vacuoles, large nuclei and which contained numerous starch granules, and elongated cells with large vacuoles, which appeared transparent and contained very few or no starch grains. Moreover, the cultures were heterogeneous since they contained single cells, multicellular aggregates (0.1– 0.5 mm diameter) and large clumps (more than 0.5 - 1.0 mm diameter). The large clumps were removed by sieving the suspension through 500 µm stainless steel sieve and dispensing 1 mL of cells (SCV) into 250 mL of flasks with 50 mL of the medium. To ensure that the cultures contained denser cell aggregates, the suspensions were allowed to settle for few minutes during subculture which enabled the large vacuolated cells to be removed using a pipette (since the dense cells settled first). Three to four months from the time of initiation, suspension cultures were largely producing cytoplasmically dense cell aggregates which were suitable for regeneration and as a target tissue for transformation experiments (see Chapter 6). However, after seven months, the cell lines began to vary in their proliferation rate. Whereas some cell lines doubled in volume within two weeks, others doubled in volume after one month. There was also a change in cell type; fast growing suspensions contained small, dense, isodiametric cells (Figure 5.2A) whereas the slow growing ones contained a high proportion of large, vacuolated cells (Figure 5.2B). Further, slow growing lines began to change colour from bright or light yellow to white. The fast growing, yellow cultures were maintained while the slow growing white cultures were discarded.

The use of higher concentrations of glutamine (400 and 800 mg/L) improved the proliferation of suspension cultures. However, this also had a negative effect on regeneration with the cells turning necrotic two weeks after plating on EM (Figure 5.3A-5.3C). A similar negative effect on regeneration was observed by reducing the concentration of sucrose from 30 g/L to 20 g/L (Figure 5.3D-5.3F). Since suspension cultures maintained in CMM containing glutamine (100 mg/L) and sucrose (30 g/L) had the highest regeneration rate, this medium was used in all subsequent experiments and is referred to as liquid callus maintenance medium ($CMM_L$).

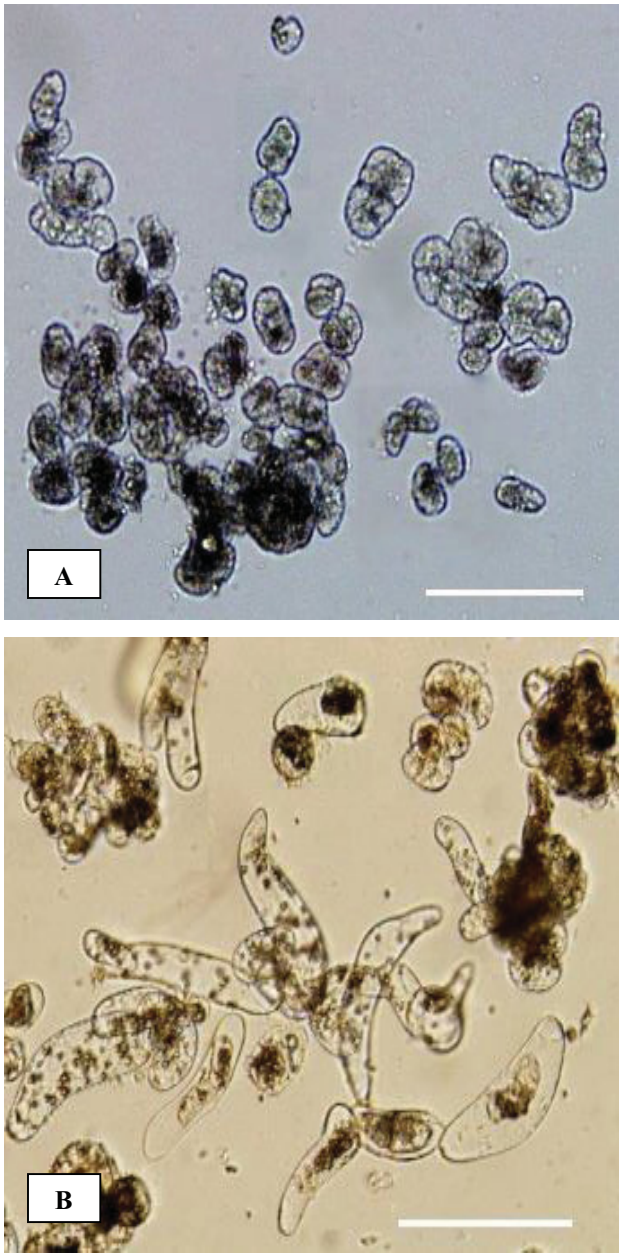


**Figure 5.2:** Change in cell types in embryogenic suspension cell culture of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. A high proportion of small, dense, isodiametric cells were present in highly prolific suspension cultures (A) whereas elongated highly vacuolated cells were present in slow growing suspension cultures (B). Scale bar = 200 µm.

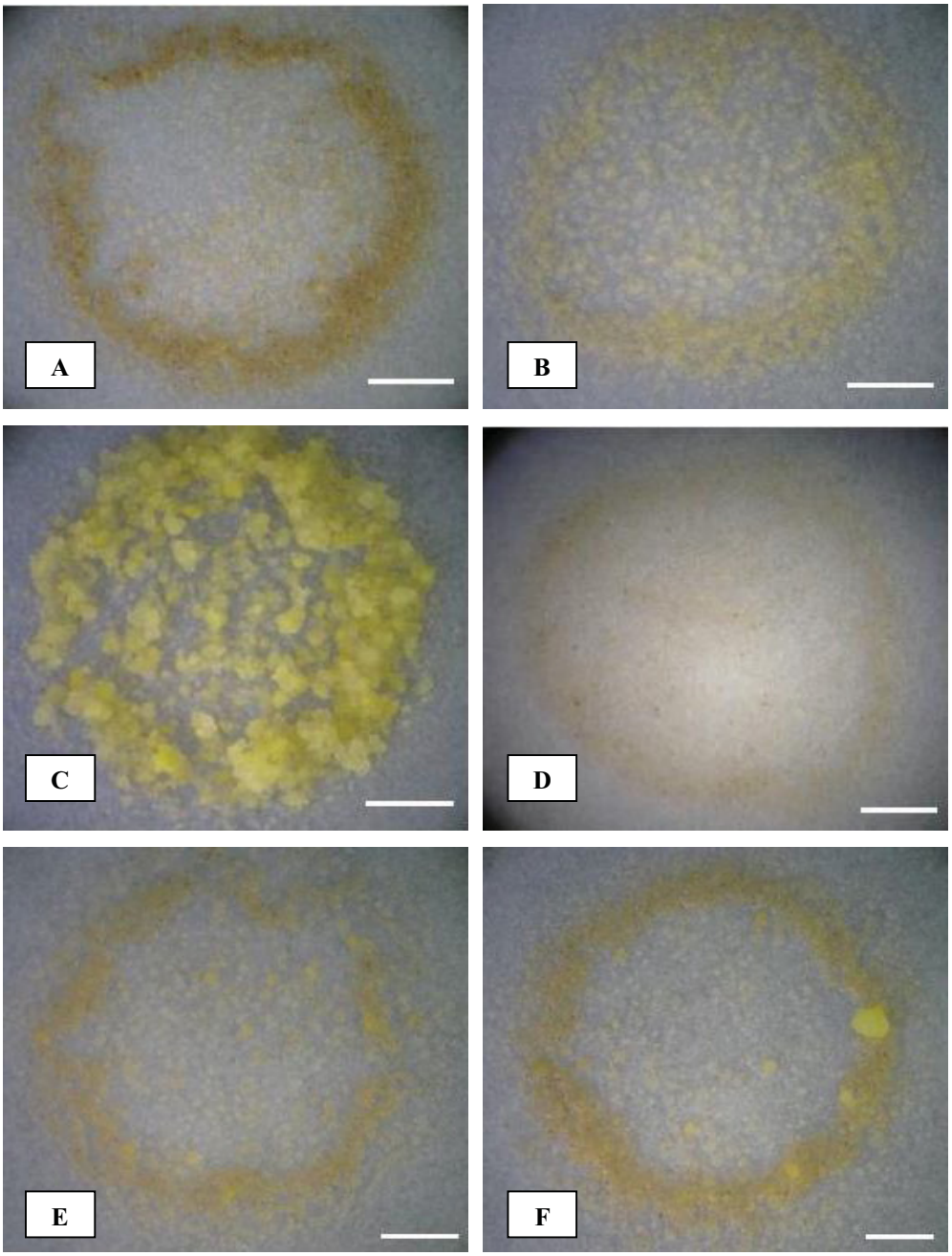


**Figure 5.3:** The effect of glutamine and sucrose concentration on regeneration from suspension cell cultures of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Suspension cells were cultured in CMM for three weeks containing either sucrose 30 g/L and glutamine at: (A) 800 mg/L, (B) 400 mg/L, (C) 100 mg/L, or sucrose 20 g/L and glutamine at: (D) 800 mg/L, (E) 400 mg/L, (F) 100 mg/L before plating onto regeneration medium. (Scale bar = 5 mm).

When callus was taken directly from the original explants on CIM and inoculated into $CMM_L$, some callus was observed to enlarge while others turned necrotic after two weeks. However, when callus was cultured on $CMM_S$ for more than two months with monthly subcultures prior to transfer into $CMM_L$, suspensions were formed at a very high frequency (~100% of callus formed suspension).

### 5.3.3 Growth curve

The growth of suspension cultures, as measured by settled cell volume (μL) per flask, displayed a relatively sigmoidal-shaped growth curve (Figure 5.4). Following an initial short lag phase (2 days), the settled cell volume began to increase from day 3 and reached a plateau on day 7. This was followed by a stationary phase which lasted up to day 10 before another increase in volume from days 11 to 14. During the initial growth phase (days 3-7), the cells settled within the allocated time of 30 minutes whereas in the later growth phase (days 11-14), the settling period was longer than one hour. Microscopic examination of these cells during the growth phases showed changes in cell type; during the first growth phase high proportion of cell aggregates were cytoplasmically dense (Figure 5.5A), with large vacuolated cells beginning to dominate the culture in the second growth phase (Figure 5.5B).

### 5.3.4 Regeneration and plant development from embryogenic callus cultured on solid medium

Embryogenic callus was transferred from $CMM_S$ to half-strength MS medium containing either no PGRs or 1.0 mg/L ABA or 0.2 mg/L NAA plus 0.1 mg/L kinetin and cultured in darkness for two weeks before transfer to low light (5 μmoles photons.$m^{-2}$s). The use of hormone-free medium resulted in the highest frequency of callus clumps producing somatic embryos (47 %) and, on average, the greatest number of embryos per callus clump (10 ± 1.2) (Table 5.3). The majority (70 %) of callus clumps on ABA became necrotic while 25 % turned green and formed globular structures which later developed into plantlets. In contrast, while the majority of callus clumps on medium containing NAA and kinetin survived, only approximately 6 % produced somatic embryos.

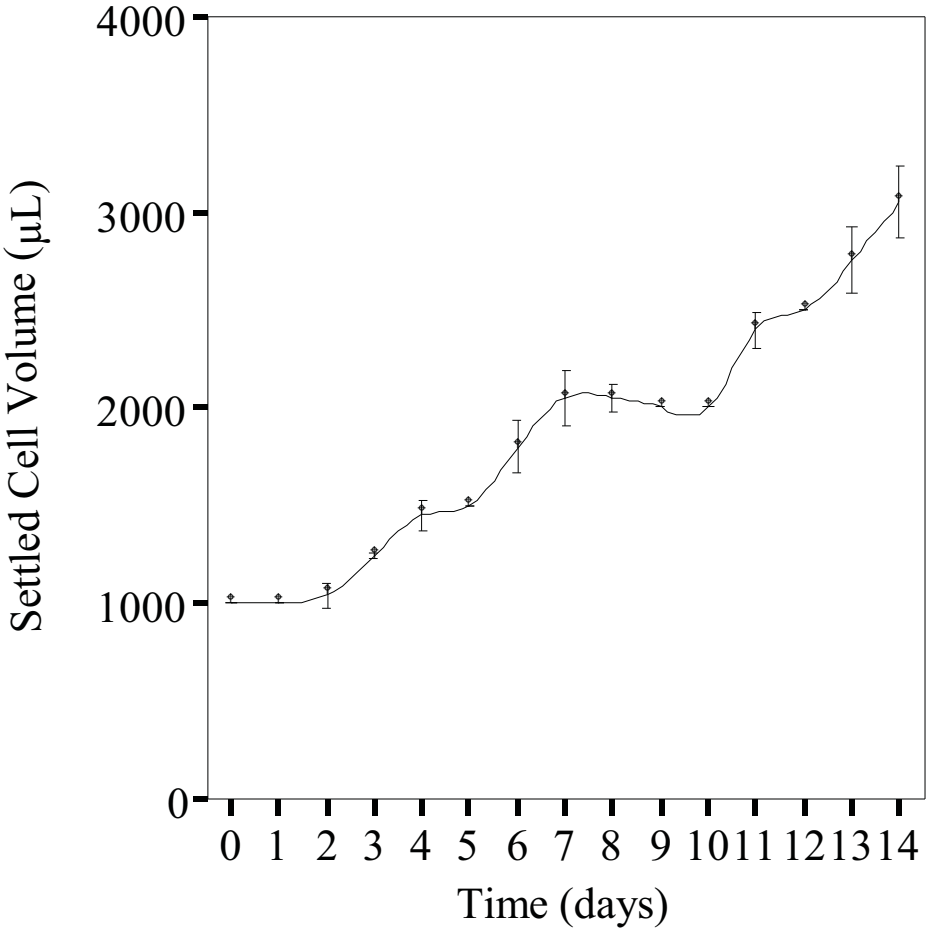


**Figure 5.4:** Growth curve of cell suspension cultures of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Each data point represents the mean of 5 replicate flasks. Error bars represent SEM.

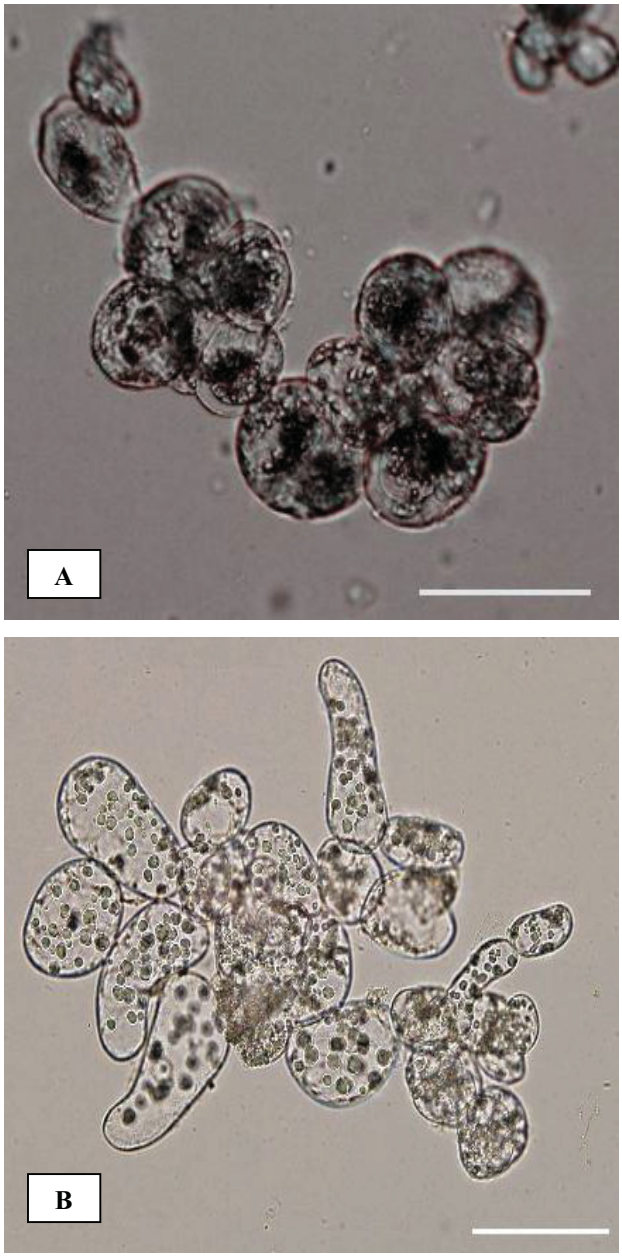


**Figure 5.5:** Morphology of taro suspension cells during the first and second growth phases (days 4 and 12, respectively). A high proportion of spherical, cytoplasmically dense cell clusters dominated at day 4 (A) whereas the cells with large vacuoles and few starch granules characteristic of non-regenerable cells began to dominate at day 12 (B). Scale bar = 100 µm.

**Table 5.3:** The effect of growth regulators on plantlet regeneration from embryogenic callus of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK on solid medium.

| Regeneration medium | Number of callus pieces inoculated | % Total callus pieces producing somatic embryos | Total number of somatic embryos produced | Mean number of somatic embryos per callus piece | Total number of plantlets produced | % Conversion | Total number of plantlets acclimatized | % of acclimatized plants survived |
|---|---|---|---|---|---|---|---|---|
| ½ MS | 49 | 47 | 160 | $10 \pm 1.2^{a}$ | 113 | $71.8^{a}$ | 70 | 100 |
| ½ MS + ABA | 56 | 25 | 52 | $3.9 \pm 0.6^{b}$ | 20 | $33.3^{b}$ | 20 | 100 |
| ½ MS + NAA + KIN | 70 | 5.7 | 50 | $6.1 \pm 3.2^{ab}$ | 38 | $32.4^{b}$ | 30 | 100 |

Concentrations of ABA, NAA and KIN in medium were 1.0 mg/L, 0.2 mg/L and 0.1 mg/L, respectively.
Values with means ± SEM are derived from of seven replicate Petri dishes with 7-10 callus pieces per replicate.
Means followed by the same letters were not significantly different ($p<0.05$).

Somatic embryos left on regeneration medium as described above began to germinate spontaneously approximately 30 days after formation and continued to germinate progressively for 60-80 days. The germination rate on hormone-free medium was approximately 70 % compared with only ~30 % using the other two media. These results showed that hormone-free medium was suitable for both somatic embryo formation and germination from embryogenic callus; this medium is henceforth referred to as regeneration medium (RM).

### 5.3.5 Regeneration and plantlet development from suspension cells

Suspension cells plated on RM did not form embryos but instead the cells proliferated slightly then turned necrotic after one to two months (Figure 5.6A). Therefore, an intermediate embryogenesis medium (EM) was developed. Four EMs were tested for their efficacy in initiating somatic embryos from suspension cells (Table 5.4). Suspension cells were dispensed in 250 uL aliquots on 70 mm Whatman filter paper and placed on the various EMs in Petri dishes and maintained in the dark. After three weeks, the large vacuolated cells became necrotic while the dense cells proliferated and formed proembryogenic masses (PEMs). Further, the yellow cell aggregates formed PEMs whereas their white counterparts only formed soft, white, watery callus. Globular structures began to form on the surface of PEMs after three weeks and continued to do so for two months (Figure 5.6B). Histological studies showed somatic embryos contained both shoot and root meristems (Figure 5.7A) and were attached to PEM through a suspensor (Figure 5.7B) and were most likely derived from the epidermal cells of PEMs.

The frequency of somatic embryo formation was significantly higher on $EM_2$ (25 ± 4.3) compared with the other media (Table 5.4). Although cells proliferated profusely on $EM_1$, very few formed embryos. The use of $EM_3$ resulted in poor proliferation and formation of PEMs with most of the cells becoming necrotic. In contrast, although cells proliferated on $EM_4$, they mostly developed into soft watery callus. After two months, the embryos were transferred to RM but all became necrotic after two months. Therefore the effects of various concentrations of sucrose and biotin on

embryo formation and regeneration were examined (Table 5.5) and the $EM_2$ was further optimized. In general, increasing the concentration of sucrose in $EM_2$ from 30 to 50 g/L significantly increased the frequency of somatic embryo formation with the greatest number of embryos formed using 50 g/L sucrose. The addition of biotin at 1.0 mg/L to $EM_2$ containing 40 g/L sucrose resulted in a significantly higher frequency of somatic embryos comparable to 50 g/L (154 compared to 137, respectively). Embryo formation was non-synchronous with various stages of embryo development being observed at the same time. Embryos were transferred to half-strength MS containing 0.05 mg/L BAP and 0.1 mg/L IAA (GM), maintained in the dark for two weeks then exposed to low light intensity to allow maturation and germination. In a separate germination experiment, there was no significant difference between the frequency of embryos germinating on GM and RM (data not shown). However, embryos on GM started to germinate after one month while those on RM took more than two months. Consequently, germination was done on GM. The frequency of germination was higher (58 %) in the embryos formed on EM containing 50 g/L sucrose followed by 40 g/L sucrose (40 %) with 40 g/L sucrose plus 1 mg/L biotin and 30 g/L sucrose having the lowest frequency (32 %) (Table 5.5). During the transfer, it was observed that individual embryos were closely associated and did not separate. After three weeks, embryos began to enlarge and turn from translucent to opaque. They subsequently turned green and germinated within two to three months (Figure 5.8A, 5.8B).

### 5.3.6 Plant recovery

The globular structures observed in this study were previously referred to as somatic embryos based on their translucent nature and the presence of a distinct epidermis. Further evidence to support this claim was provided by the observation that the structures appeared to be bipolar as both shoots and roots formed simultaneously when germinating (Figure 5.9A). As multiple somatic embryos were left intact on callus clumps/PEMs and not separated for germination, germinated embryos tended to be fused into clumps of plantlets (Figure 5.9B). Individual plantlets were separated, and transferred into 28 mL McCartney bottles containing 10 mL of half-strength MS medium for further development (Figure 5.9C). A total of 120 plantlets were

generated which, upon reaching a height of 4-5 cm, were transferred to potting mix (consisting of soil and perlite in a 1:1 ratio) and acclimatized in a screen house. All 120 plantlets survived the acclimatization process, continued to grow and, in comparison to the mother plant, appeared phenotypically normal after two months. (Figure 5.9D).

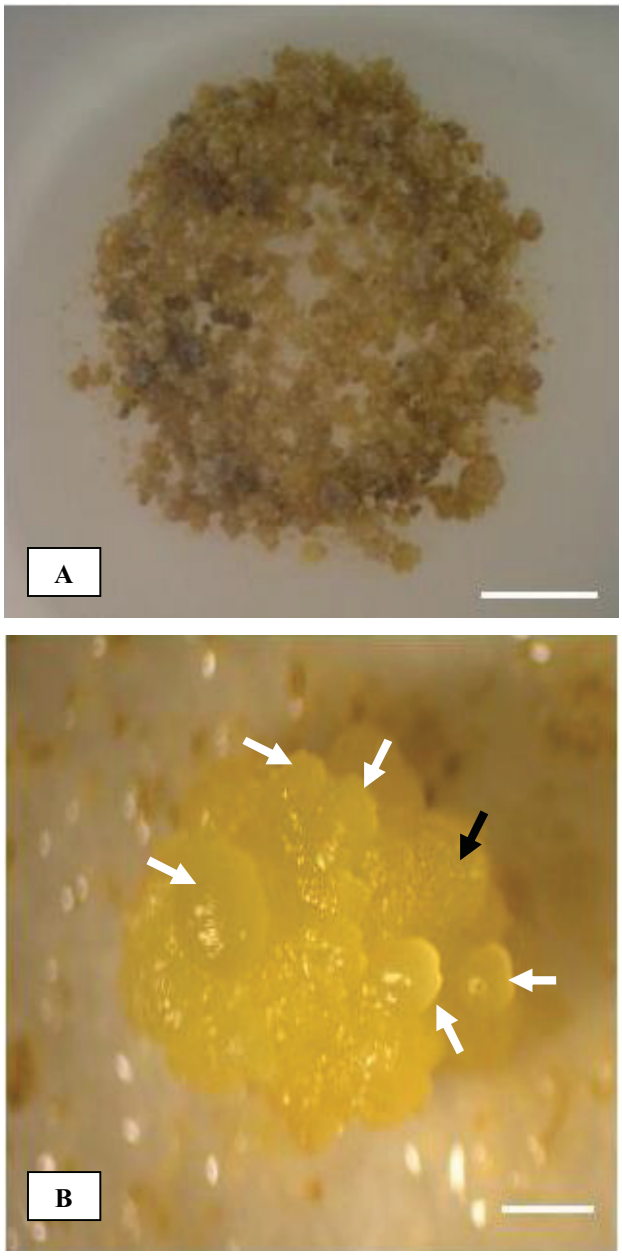


**Figure 5.6:** Formation of PEMs and somatic embryos from embryogenic suspension cells of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Suspension cells proliferated, and turned necrotic on RM (A) whereas on EM they formed PEMs (black arrow) with globular, translucent embryo-like structures (white arrows) forming on the surface of PEMs (B). Scale bar = 5 mm (A), 2 mm (B).

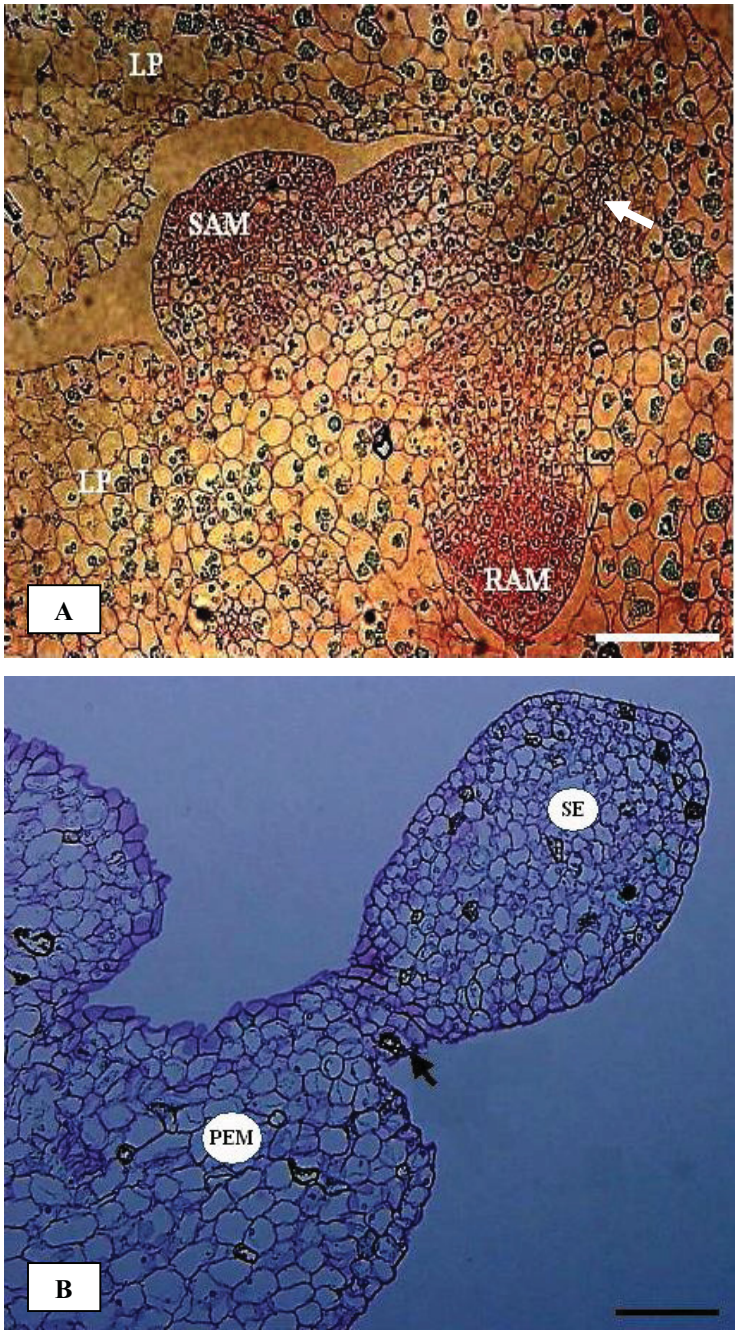


**Figure 5.7:** Histology of mature and immature somatic embryos derived from cell suspension cultures cv. CPUK. A mature somatic embryo with shoot meristem (SAM) between leaf primordia (LP), root meristem (RAM) and vascular tissue (white arrow) (A); (B) a globular somatic embryo (SE) attached to proembryogenic mass (PEM) via suspensor (black arrow). Scale bar = 100 μm.

**Table 5.4:** The effect of growth regulators on taro (*C. esculenta* var. *esculenta*, cv. CPUK) somatic embryo initiation following transfer of suspension cells to embryogenesis medium (EM). Suspension cells were dispensed in 250 µL aliquots on Whatman filter paper discs placed on various embryogenesis media in Petri dishes and maintained in the dark for two months for embryo formation.

| Embryogenesis Medium | Plant Growth Regulators (mg/L) | Mean total number of somatic embryos produced per replicate |
|---|---|---|
| $EM_1$ | TDZ + 2,4-D (1.0) (0.5) | $2.1 \pm 0.82^{a}$ |
| $EM_2$ | TDZ + 2,4-D (0.1) (0.05) | $25 \pm 4.3^{b}$ |
| $EM_3$ | TDZ + 2,4-D (0.01) (0.005) | $0.6 \pm 0.43^{c}$ |
| $EM_4$ | Zeatin + NAA (0.1) (0.05) | $1.4 \pm 0.62^{ac}$ |

Values with the means ± SE are derived from 10 replicate Petri dishes with 50 µL settled cell volume of suspension cells per replicate. Means followed by the same letter were not significantly different ($p < 0.05$).

**Table 5.5:** Effect of sucrose and biotin on the frequency of embryo formation and germination from embryogenic suspension cells of *C. esculenta* var. *esculenta* cv. CPUK. Suspension cells were plated on embryogenesis medium ($EM_2$) containing various concentrations of sucrose (30, 40, 50 g/L) or 40 g/L sucrose + 1.0 mg/L biotin and maintained in the dark for two months followed by transfer of SEs to germination medium (GM).

| Sucrose concentration in medium (g/L) | Mean total number of embryos produced per replicate | Total number of embryos transferred to germination medium | Total number of embryos germinated | % Germination |
|---|---|---|---|---|
| 30 | $58 \pm 3.6^{a}$ | 300 | 96 | 32 |
| 40 | $60 \pm 4.8^{a}$ | 260 | 105 | 40 |
| 50 | $137 \pm 15^{b}$ | 440 | 256 | 58 |
| 40 + Biotin (1.0 mg/L) | $154 \pm 19^{b}$ | 740 | 233 | 32 |

Values with the means ± SEM are derived from 10 replicate Petri dishes with 50 µL settled cell volume of embryogenic suspension cells per replicate. Means followed by the same letters are not significantly different ($p<0.05$).

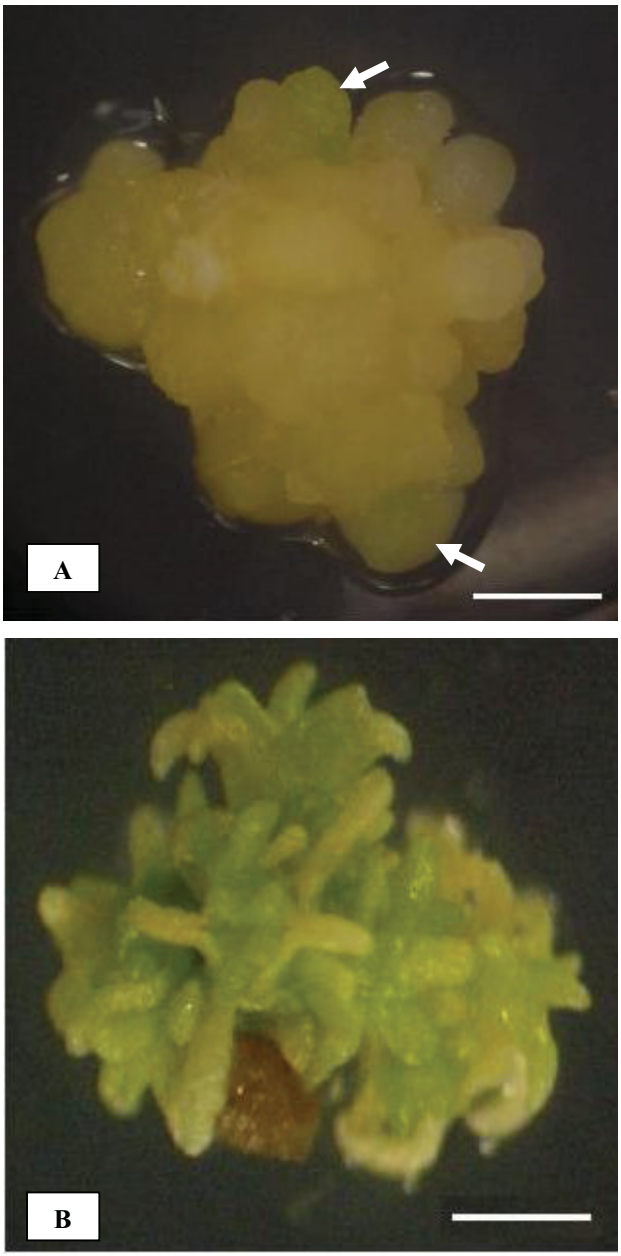


**Figure 5.8:** Maturation and germination of somatic embryos from suspension cultures of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Upon transfer to GM (A) embryo formation continued while the existing somatic embryos began to turn opaque and then turned green after two months (arrows). Germination commenced after two months (B). Scale bar = 2 mm.

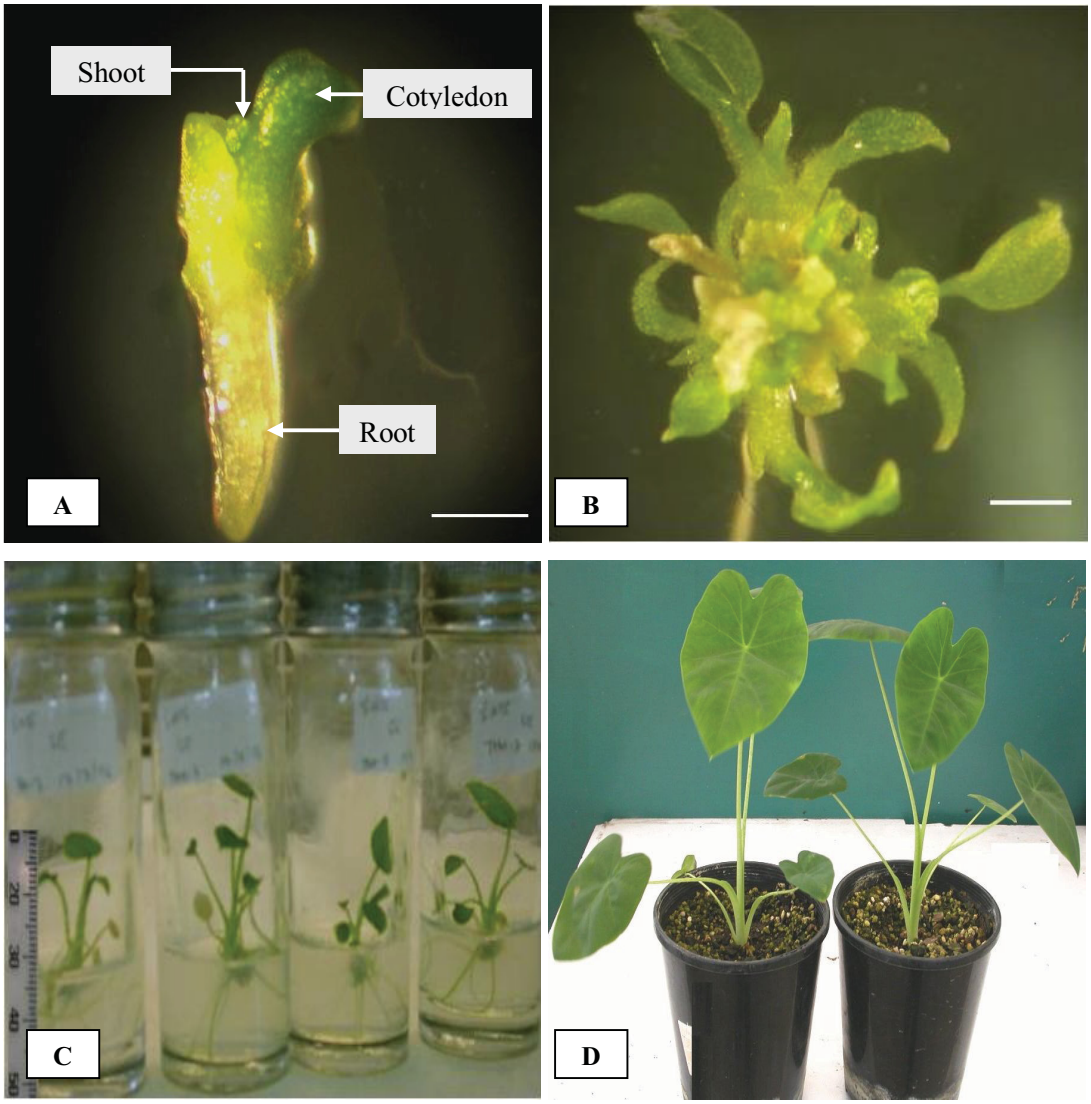


**Figure 5.9:** Regeneration of taro plants from somatic embryos cv. CPUK. (A) Early germinating somatic embryos showing shoot and root poles, (B) Clump of fused somatic embryos germinating, (C) Germinated somatic embryos after transfer to individual culture vessels for further development and (D) Acclimatized somatic embryo-derived plants. (Scale bar =2 mm)

## *5.4 Discussion*

Callus proliferation is an integral stage for any efficient regeneration system since it ensures a regular supply of plant tissue which obviates the need to constantly reinitiate new cultures. The maintenance medium established for taro in this study promoted good growth and proliferation of callus while maintaining its embryogenic capacity. Thus, half-strength MS medium fortified with TDZ (1.0 mg/L), 2,4-D (0.5 mg/L) plus glutamine (800 mg/L) was established as callus maintenance medium in *C. esculenta* var. *esculenta*. The percentage of callus pieces proliferating increased by 4–5 fold on medium containing both 2,4-D and TDZ in comparison with medium containing either of these growth regulators individually. The inabilities of 2,4-D and TDZ to foster proliferation of embryogenic callus independently emphasizes the significance of an optimal balance of both auxin and cytokinin for callus growth and proliferation since both cell division and cell expansion occur in actively dividing embryogenic callus (Mineo, 1990). It is known that both auxin and cytokinin promote cell expansion and cell division. A high auxin to cytokinin ratio promotes root development, a high cytokinin to auxin ratio promotes shoot development while an intermediate cytokinin to auxin ratio promotes callus growth and development. In this study, this balance was achieved with the combination 1.0 mg/L TDZ and 0.5 mg/L 2,4-D. In a similar study, a low concentration of cytokinin in combination with an even lower concentration of auxin resulted in proliferation of embryogenic callus in both chestnut and the evergreen tree *Castanopsis sieboldii* (Vidhanaarachchi *et al.*, 2004).

The incorporation of glutamine at 800 mg/L in the callus maintenance medium significantly increased the mean fresh weight of callus produced without considerably affecting the rate at which the callus proliferated. Further, the presence of glutamine also maintained the regeneration capacity of the callus. In the absence of glutamine, approximately 40 % of the callus became soft, watery and non-regenerable within three to four weeks. In contrast, the inclusion of glutamine resulted in the callus remaining regenerable for over a month. However, higher glutamine concentrations (1600 and 2400 mg/L) appeared to have a toxic effect and caused a reduction in both

the frequency of proliferation and the mean fresh weight of callus. Glutamine has also been used to improve the proliferation of embryogenic callus in *Douglas fir* (Kirby, 1982), *Pinus pinaster* (David *et al.*, 1984) and *Cryptomeria japonica* (Ogita *et al.*, 2001). Glutamine readily increases the amount of available nitrogen which enhances the synthesis of certain macromolecules or metabolites (Ogita *et al.*, 2001) while maintaining inorganic nitrogen at a low concentration.

In this study, the effect of glutamine was found to be dependent on whether the media was liquid or solid. For example, embryogenesis was inhibited in cells derived from liquid medium containing 800 mg/L glutamine with all cells turning necrotic after two to three weeks following transfer on hormone-free medium. In contrast, the same concentration in solidified callus maintenance medium was not inhibitory. The inhibitory effect of glutamine in liquid cultured cells was eliminated by reducing the concentration to 100 mg/L. Glutamine is chemically unstable and degrades rapidly in nutrient medium to produce ammonia which is toxic to cultured cells (Barrett *et al.*, 1997; Gorret *et al.*, 2004). It is possible, therefore, that degrading glutamine is providing excessive nitrogen which has an inhibitory effect on regeneration even after transfer to glutamine-free media. It has been reported that a low ratio of ammonium ion ($NH^+_4$) to nitrate ($NO^-_3$) (1:2 or 1:4) promoted high regeneration in *Prunus domestica* callus (Nowak *et al.*, 2007). Similarly, soybean embryogenic culture lost its regeneration capacity when a 1:0 ratio was used while a ratio of 1:4 favoured regeneration (Samoylov *et al.*, 1998). The lower level of glutamine in liquid medium would have released less ammonia, which could have been assimilated by the growing cells. The observed non-inhibitory effect of high glutamine concentration to cells on solid medium may have been due to its reduced availability or slower degradation.

Decreasing the sucrose concentration in suspension cultures also inhibited regeneration. Sucrose provides energy to the growing cells, however as cells were capable of growing in liquid medium containing 30 g/L sucrose for 14 days without subculture, this reduction in regeneration may not be a simple case of energy

limitation. Sucrose may also be acting as an osmoticum affecting the physiology of the cells.

Consequently, CMM were established for *C. esculenta* var. *esculenta* which comprised (i) agar-solidified CMM ($CMM_S$) containing ½ MS, TDZ (1.0 mg/L), 2,4-D (0.5 mg/L), sucrose 30 g/L and glutamine (800 mg/L) and (ii) liquid CMM ($CMM_L$) having the same components as $CMM_S$ except the concentration of glutamine was 100 mg/L.

The inability to initiate suspension cultures using callus directly from CIM may have been due to shock from the physical isolation of callus from the mother explants. Alternatively, it could have been due the characteristics of the callus at this particular stage of development (Slater *et al.*, 2003). Transferring the callus to $CMM_S$ prior to $CMM_L$ provided a transition step for the callus to proliferate and allowed them to acclimatize to the new environment. In addition, culturing callus on $CMM_S$ improved friability (Slater *et al.*, 2003) and, as a result, it dissociated easily when agitated in $CMM_L$. Further, non-embryogenic sectors of callus formed while on $CMM_S$ which could be excluded when initiating liquid cultures. Such a selection process was also reported in maize (Swedlund and Locy, 1993) whereby monthly subculture on solid maintenance medium facilitated maintenance of regenerable callus. Failure to separate embryogenic from non-embryogenic callus often resulted in non-embryogenic callus dominating the culture (Sakhanokho *et al.*, 2001) and caused a reduction in the regeneration capacity of callus over time. Based on this observation, a similar phenomenon was expected in suspension cultures. Therefore, during each subculture, suspension cultures were agitated vigorously and allowed to settle for 3-5 minutes. The lighter non-embryogenic cells and cell aggregates were removed with a pipette while the settled dense, embryogenic cell aggregates were preserved.

Taro suspension cultures were maintained in an embryogenic state by weekly subculture. In preliminary experiments (data not shown) subculturing at 10 –12 day intervals led to necrosis of the culture. This may have been due to nutrient and/or

growth regulator(s) depletion or accumulation of secondary metabolites or other cellular waste (Liu *et al.*, 1998). Frequent subculture at short intervals has been reported as a practical solution to overcome this problem (Srangsam and Kanchanapoom, 2003). However, this may also have a negative effect on culture survival (Schween *et al.*, 2003) possibly due to inadvertent removal (through serial dilution) of growth promoting substances accumulating in the cultures (van Hengel *et al.*, 2001; Umehara *et al.*, 2004). During subculture in this study, therefore, 1 mL of settled cell volume (SCV) of suspension cells and 10 mL of the conditioned medium were added to 40 mL of fresh $CMM_L$.

The volume of suspension cells inoculated at subculture has been reported to affect the growth rate of suspension cultures. For example, the inoculation of 1-2 mL (1.5 – 3.0 %) SCV into new suspension cultures was sufficient for good growth and regeneration in banana (Strosse *et al.*, 2003). Similarly, use of 2.0 % of SCV inoculum in taro resulted in a fortnightly two-fold multiplication rate. In the present study, a very low inoculum (0.25 and 0.5 mL SCV) (data not shown) resulted in low growth rate while a very high density was not tested. This demonstrates that there is a critical cell density required for adequate proliferation. The effect of culture density on regeneration was not determined in this research with a low culture density always maintained.

The growth kinetics of suspension cells showed that there was a lag phase of two days, an exponential phase between days 2-7, and a plateau after day 7. The lag phase is an adaptive period in which the cells acclimatize to their new environment. The exponential phase is the period of maximum cell division and growth leading to a dramatic increase in the number of cells. This is followed by a stationary phase in which the rate of cellular division is gradually reduced due to depletion of nutrients and accumulation of toxic or growth inhibiting substances produced by damaged and dead cells (da Silva *et al.*, 2005). Although settled cell volume does not usually change after the stationary phase, this was not the case with taro in which the cell volume was found to increase. However, the characteristics of these cells indicated

they were non-regenerable and, as such, cells from the end of the first exponential growth phase (a seven-day subculture) was selected for further studies.

In general, highly prolific cultures tend to lose regenerability (Ikeda-Iwai *et al.*, 2002). As a result, maintaining a low cell culture density and prolonging subculture times have been suggested as methods for maintaining regenerability (Guo and Zhang, 2005). In contrast, rapidly growing taro suspension cells were found to be regenerable in this study unlike slow growing cells. The highly prolific suspension cultures doubled in cell volume fortnightly and contained a large proportion of cells with embryogenic characteristics namely cells that were small, cytoplasmically dense, isodiametric in shape and often present in small multicellular clumps. Such cultures appeared cream/yellow. In contrast, slow growing suspension cultures took over a month to double in cell volume, contained a high proportion of large, highly vacuolated, non-embryogenic cells and appeared white. Further, the yellow cell aggregates formed PEMs and SEs on EM while the white cell aggregates formed white, soft and watery callus. Therefore, cultures that changed from bright or light yellow to white and exhibited a very slow growth rate after six to seven months were discarded.

Regeneration involves the differentiation and germination of somatic embryos and subsequent recovery of viable plants. As the callus initiation and proliferation steps were conducted in the dark, the regeneration step was done at low light intensity (5 μmoles photons.$m^{-2}$s) to avoid possible shock in the transition to light culture required for plantlets. Embryogenic callus was glossy, compact, pale yellow and had small globular embryo-like structures. The pathway of regeneration was thought to be embryonic based on (i) the structures being translucent, globular and having a distinct epidermis, (ii) the simultaneous formation of shoots and roots when germinating and (ii) histological studies which revealed the presence of both shoot and root meristems.

In general, the basic regeneration media used for many species is similar to the callus initiation/proliferation media, except that growth regulators are omitted or used at very low concentrations. Regeneration in *C. esculenta* var. *antiqurum* has been

achieved using medium containing NAA and kinetin (Abo El-Nil and Zettler, 1976) and in *C. esculenta* var. *esculenta* using (i) taro corm extract (TE) plus 2,4,5-T and (ii) taro corm extract plus coconut water (CW) for shoots and roots formation respectively (Yam *et al.*, 1990). In this study the presence of NAA and kinetin or ABA in the regeneration medium appeared to interfere with embryo formation, maturation and germination processes. Thus, embryo formation, maturation and germination from taro callus on solid media was achieved in a single step on hormone free half-strength MS medium without the addition of complex organic additives such as TE and CW. In contrast, regeneration from suspension cells required successive steps. No embryos formed on hormone-free media but instead the cells became necrotic after one to two months. However, when cells were plated on embryogenesis medium (EM) containing 0.1 mg/L TDZ and 0.05 mg/L 2,4-D, the cells proliferated and formed PEMs with globular somatic embryos forming on their surface. These embryos were attached to PEMs via a suspensor-like structure and appeared to form from the epidermal and sub-epidermal cells of the PEMs. A similar phenomenon was also reported in ginger (Guo and Zhang, 2005). One of the critical events leading to the formation of somatic embryos is the establishment of cell polarity, which can result from an auxin concentration gradient when callus is transferred to medium with low or no auxin (Souter and Lindsey, 2000). Such a gradient may be established as a result of endogenous auxin synthesis or by the provision of exogenous auxin (Ribnicky *et al.*, 1996). It would appear that taro suspension cells could not synthesize and accumulate the required level of endogenous IAA and/or other cellular metabolites required for the formation of globular embryos. As a consequence, the application of very low concentrations of exogenous 2,4-D and TDZ was necessary. Both of these growth regulators have been reported to modulate endogenous auxin (Visser *et al.*, 1992; Ribnicky *et al.*, 1996; Panaia *et al.*, 2004).

An increase in sucrose concentration (30-50 g/L) in the embryogenesis medium was shown to enhance the frequency of somatic embryogenesis from suspension cells. A high frequency of embryogenesis at high sucrose concentrations has also been reported in maize (Kamo *et al.*, 1985), cucumber (Lou and Kako, 1995), sugar cane (Blanco *et al.*, 1999; Gandonou *et al.*, 2005) and melon (Nakagawa *et al.*, 2001). At

these concentrations, the action of sucrose is likely to be as an osmoticum or other developmental regulator rather than a carbon source. The combination of biotin (1.0 mg/L) with sucrose (40 g/L) increased embryo formation by 2.6-fold when compared with the numbers formed on 40 g/L sucrose alone. Biotin is important in carboxylation reactions and regulating genes involved in synthesis of some fatty acids, and development of plant embryos (Wurtele and Nikolau, 1992). The stimulating effect of biotin on embryogenesis has also been reported in date palm (Al-Khayri, 2001). Further, it has also improved embryo development in carrot (Wurtele and Nikolau, 1992) and has been shown to restore normal seedling germination and development in *Arabidopsis* mutant *bio1*, which could not synthesize biotin (Che *et al.*, 2003).

Following embryo development and early maturation, further maturation and germination was achieved by complete removal of 2,4-D and TDZ from the media while maintaining very low concentrations of BAP (0.05 mg/L) and IAA (0.1 mg/L). However, a large number of embryos did not develop past the globular stage indicating that production of a large number of embryos does not always translate into a high regeneration rate. Thus, further studies are required to refine the parameters affecting embryo maturation and germination.

The optimized somatic embryogenesis protocol for taro including initiation (Chapter 4), callus multiplication and regeneration (Chapter 5) is summarized in Figure 5.10.

In summary, an effective callus maintenance medium ($CMM_S$) for taro was developed and embryogenic callus was successfully multiplied and maintained for over a year without losing its regenerability. The highly regenerable and rapidly growing suspension cell cultures established in $CMM_L$ represent an ideal target tissue for the genetic transformation of this plant (Chapter 6).

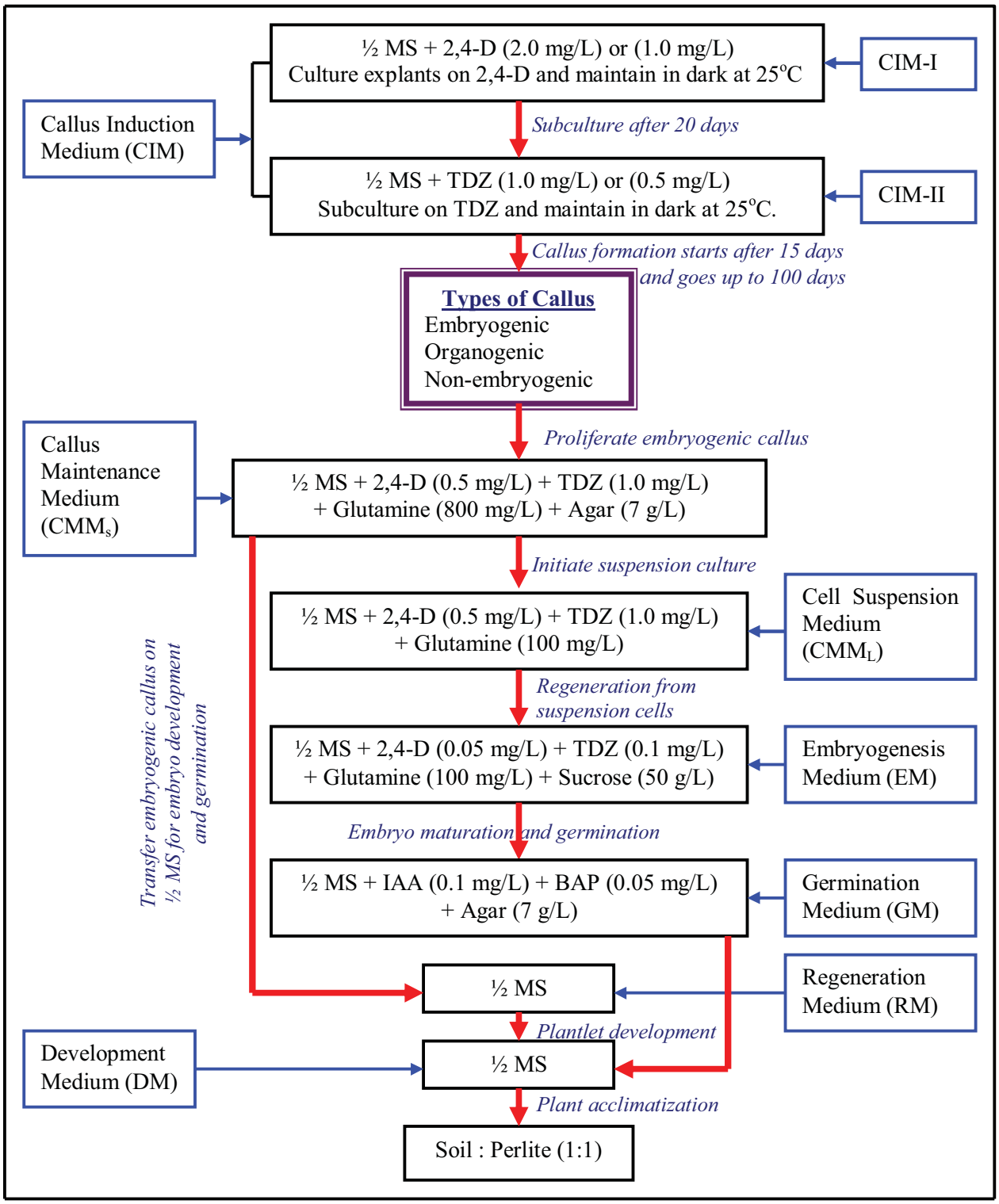


**Figure 5.10:** Flow chart depicting the optimized protocol for somatic embryogenesis in taro (*Colocasia esculenta* var. *esculenta*)

# CHAPTER 6

## *Agrobacterium* and Microprojectile Bombardment-Mediated Transformation of Taro (*Colocasia esculenta* var. *esculenta*)

### *6.1 Introduction*

Many pests and diseases limit taro cultivation causing significant trade losses and jeopardizing food security. The improvement of existing taro germplasm can potentially be achieved by both conventional breeding and biotechnology approaches. However, conventional breeding is a lengthy process (Wilson, 1990) and is hampered by a paucity of resistant germplasm. Further, amongst different ethnic groups there are preferences for certain cultivars of taro. Unlike conventional breeding, genetic transformation provides a means of introducing only one or a few genes to an already accepted cultivar. The source of these genes is not necessarily restricted to taro and can be derived from other plant species or completely different organisms. Thus the development of an efficient transformation system for taro could greatly benefit taro production.

Production of improved plant varieties via genetic transformation offers an attractive alternative to conventional breeding. Transformation of some agronomically important monocotyledonous crops such as sugar cane (Bower and Birch, 1992), banana (Becker *et al.*, 2000; Khanna *et al.*, 2004), maize (O'Kennedy *et al.*, 2001), wheat (Jones, 2005) and rice (Riaz *et al.*, 2006) has been successfully achieved using both *Agrobacterium tumefaciens* and microprojectile bombardment. Genetic transformation in *Colocasia esculenta* var. *esculenta*, however has been largely neglected possibly due to the difficulties in developing an efficient regeneration system or the lack of focus on a crop of low significance to developed nations where a large portion of funding and expertise resides. There is only one report on transformation in *Colocasia esculenta* var. *esculenta* via microprojectile bombardment (He *et al.*, 2004) and one report on *Agrobacterium tumefaciens*–

mediated transformation (He *et al.*, 2008). The efficiency of transformation via biolistics was reported to be very low with only one stably transformed plant generated. The same research group achieved a slightly higher frequency of transformation via *Agrobacterium* where six stable transgenic plants were generated.

The development of a plant transformation system requires a method of transferring genes into plant cells, a gene conferring the useful new trait together with a promoter directing the appropriate level and pattern of expression, an effective selective agent to suppress the growth of non-transformed cells and the ability to regenerate plants from single transformed cells (Sharma *et al.*, 2005). Chapters 4 and 5 of this thesis described the development of an effective taro somatic embryogenesis system including regenerable embryogenic suspension cell cultures. Such cell cultures are considered to be an excellent target tissue for genetic transformation since they (i) can be easily proliferated and thus provide ample target tissue, (ii) consist of small cell clumps allowing maximal exposure to the transforming agent thereby facilitating the identification of independent transformation events within the dispersed cell clusters under selection and (iii) allow the recovery of non-chimeric transformants due to the unicellular origin of embryos (Aguado-Santacruz *et al.*, 2002; Sahrawat *et al.*, 2003; ul-Haq, 2005). Another important aspect of transgenesis is the relative activity and tissue specificity of promoters needed to control transgene expression. The activity of various promoters has only been examined previously in bombarded taro leaves (Yang *et al.*, 2003).

The research in this chapter describes the use of embryogenic suspension culture cells in the development of taro transformation protocols. The *gfp* gene, which encodes the green fluorescent protein (GFP), was selected as the reporter gene due to the non-destructive nature of the assay system while the *npt* II gene, which encodes neomycin phosphotransferase II, was chosen as the selectable marker gene due to its widespread acceptance in field releases of transgenic plants. Some of the factors affecting the efficacy of both *Agrobacterium* and biolistic mediated transformation were examined and stably transformed embryos were generated. Further, the activity of the cauliflower mosaic virus 35S (CaMV 35S) promoter was compared with the taro

bacilliform virus (TaBV-600) and maize polyubiquitin-1 gene (*Ubi*-1) promoters using the *uid*A (GUS) reporter gene.

## *6.2 Materials and Methods*

### 6.2.1 Plant material for transformation

Embryogenic suspension cultures of taro accessions, THA-07 and CPUK were maintained in liquid maintenance medium ($CMM_L$) by weekly subculture (Chapter 5) for transformation studies.

### 6.2.2 Plasmids

Schematic representations of the transformation vectors used in this study are shown in Figure 6.1. The plasmids pGem4z-TaBV-GUS, pGem4z-Ubi-GUS and pUC-CaMV 35S-GUS used for promoter activity studies were provided by Dr. Jason Geijskes (Queensland University of Technology) while the plasmid pART-TEST7 was provided by Dr. Ben Dugdale (QUT).

### 6.2.3 Kill curve

To determine the optimum level of selection for non-transformed cells, suspension cells were placed on agar-solidified $CMM_L$ containing either kanamycin (0, 50, 100, 150, 200, 250 mg/L) or hygromycin (0, 10, 20, 30, 40, 50 mg/L) with five replicates for each concentration. Cells were prepared by placing suspension cells in a graduated Falcon tube and the settled cell volume/medium ratio was adjusted to 1:5 (v/v). The cells were then resuspended and 250 µL aliquots dispensed onto 70 mm diameter Whatman filter paper placed on selection medium in 90 mm x 20 mm Petri dishes and maintained in the dark at 25 $^{o}$C. The progress was monitored weekly for two months with monthly subculture.

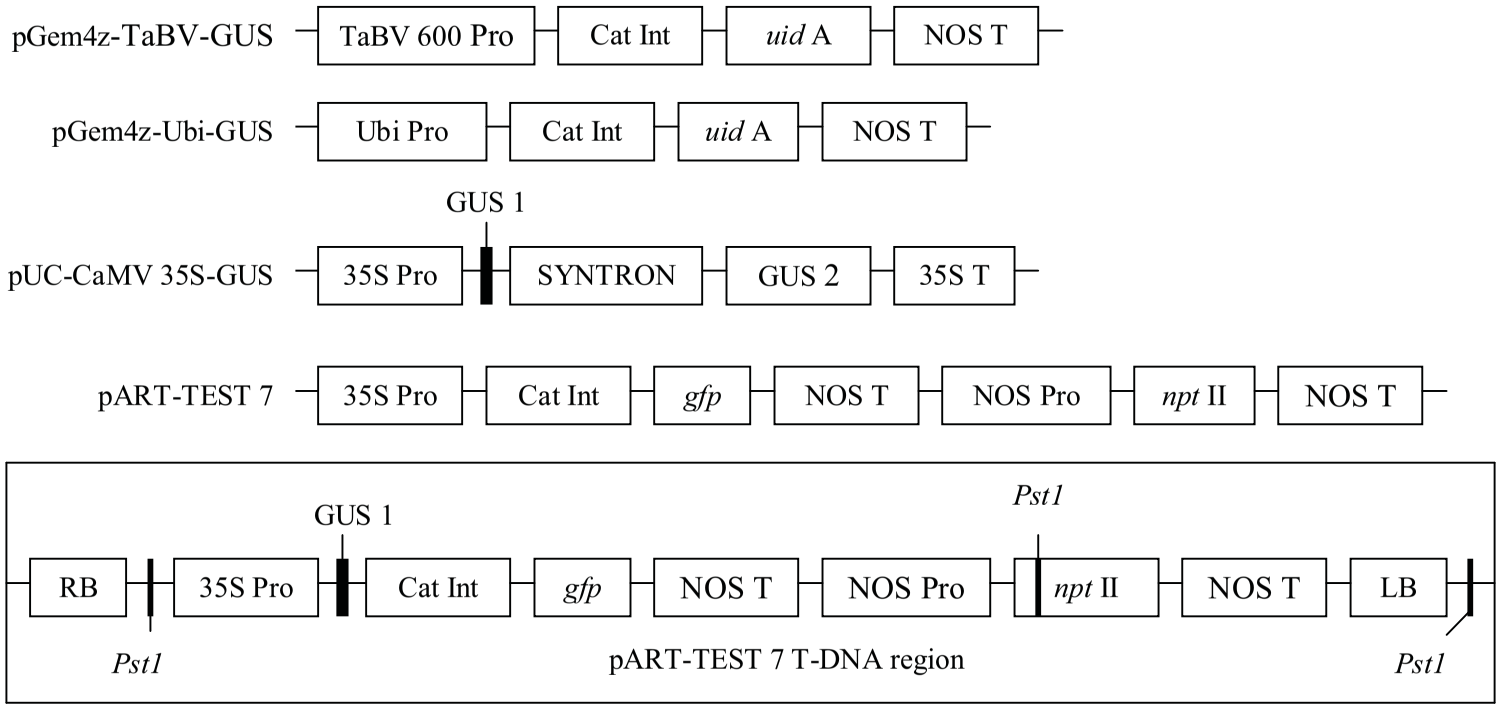




**Figure 6.1:**Schematic representation of gene constructs used for transformation in taro embryogenic suspension cells. 35S Pro = cauliflower mosaic virus 35S promoter, npt II = neomycin phosphotransferase gene, 35S T = cauliflower mosaic virus 35S 3' UTR, TaBV 600 Pro = promoter from taro bacilliform virus, Cat Int = catalase intron, uid A = β-glucuronidase reporter gene (GUS), GUS 1 = GUS first exon, SYNTRON = synthetic intron, GUS 2 = GUS second exon, NOS T = nopaline synthase 3'UTR, Ubi Pro = promoter and first intron from maize polyubiquitin-1 gene, gfp = green fluorescent protein reporter gene (GFP), NOS Pro = promoter from *Agrobacterium* nopaline synthase, RB = right T-DNA border, LB = left T-DNA border.

### 6.2.4 Microprojectile bombardment-mediated transformation

#### 6.2.4.1 Preparation of target materials

Suspension cells were harvested four days after subculture and passed through a 500 µm mesh. The filtrate was allowed to settle for 10 minutes and then sufficient supernatant was removed to leave a settled cell volume/liquid medium ratio of 1:5. Cells were then resuspended and dispensed in 250 µL aliquots onto 70 mm diameter Whatman filter paper discs in 90 mm x 15 mm Petri dishes containing agar-solidified $CMM_L$. Cells were bombarded six days after plating.

#### 6.2.4.2 Bombardment conditions

Suspension cells were bombarded using a particle inflow gun (Finer *et al.*, 1992). Gold particles (Bio-Rad) 1.0 µm in diameter were used as microprojectiles; 120 mg of gold was washed three times in ethanol followed by three washes in sterile distilled water before suspension in 1.0mL of sterile 50 % (v/v) glycerol.

To prepare microprojectiles for bombardment, 25 µL of gold particle suspension was sonicated for 30 seconds, and then mixed with 2.0 µL of 1.0 µg/µL plasmid DNA, 25 µL of 2.5 M $CaCl_2$ and 5.0 µL of 0.1 M spermidine-free base. All solutions were kept on ice. The gold was kept in suspension for five minutes by vortexing every 20–30 seconds, allowed to precipitate for 10 minutes on ice and then 22 µL of the supernatant was removed. The remaining suspension was vortexed immediately prior to using 5.0 µL aliquots of the mixture for each bombardment.

The particle/DNA mixture was placed in the centre of the syringe filter unit (swinney). Target tissue was placed 7.5 cm from the point of particle discharge and covered with a stainless steel mesh baffle with a mesh size of 270 µm. Helium pressure was 550 kPa and chamber vacuum was -85 kPa. Cells were taken for fluorometric GUS assays 72 hours post-bombardment.

### 6.2.4.3 Effect of osmoticum on the efficiency of transformation

To study the effect of osmoticum on the efficiency of transformation, suspension cells maintained on solid $CMM_L$ in the dark for six days were transferred to osmoticum medium ($CMM_L$ containing 0.2 M mannitol and 0.2 M sorbitol) and maintained in darkness for two hours before transformation. The cells were then bombarded as described above and maintained on the osmoticum for an additional two hours. Cells were then transferred to solid $CMM_L$ minus osmoticum. The cells were transferred by moving the filter paper on which they had been dispensed.

### 6.2.4.4 Promoter activity

The activities of the cauliflower mosaic virus 35S (CaMV 35S), maize polyubiquitin-1 gene (*Ubi*-1) and taro bacilliform virus (TaBV-600) promoters (Yang *et al*., 2003) were studied in taro suspension cells. The plasmids pGem4z-TaBV-GUS, pGem4z-Ubi-GUS and pUC-CaMV35S-GUS (Figure 6.1) were coated onto gold microprojectiles and bombarded into suspension cells as described above. Three independent experiments were conducted with six replicates for each promoter construct per experiment. Five replicates were used for fluorometric assays and one for GUS histochemical staining.

### 6.2.4.5 Selection and regeneration

Transformed cells were selected on agar-solidified $CMM_L$ containing kanamycin (150 mg/L). Bombarded embryogenic cells including non-bombarded controls were transferred to selection medium three days post-bombardment and maintained in the dark for two months with monthly subculture. Subculture was done by transferring the supporting filter paper to fresh medium. After two months, the cells were transferred to embryogenesis medium (EM) containing kanamycin 100 mg/L and maintained in the dark for two additional months without subculture to allow for embryo formation and maturation. After two months the PEMs together with embryos were removed from filter paper and placed directly on germination medium (GM), which contained half-strength MS, BAP (0.05 mg/L) and IAA (0.1 mg/L) minus

kanamycin and maintained in darkness for two weeks followed by incubation under low light intensity.

### 6.2.5 *Agrobacterium tumefaciens* - mediated transformation

#### 6.2.5.1 *Agrobacterium* culture

*Agrobacterium tumefaciens* (AGL1 strain) harbouring the plasmid pART-TEST7 (Figure 6.1) was prepared for inoculation by streaking the bacterial culture on Yeast-Mannitol medium (YM, Table 1) containing rifampicin (25 mg/L), spectinomycin (100 mg/L) and carbenicillin (250 mg/L). The cultures were incubated at 28 $^{o}$C for three days. Single isolated colonies were picked and inoculated into 3 mL of YM broth containing rifampicin (25 mg/L) and spectinomycin (100 mg/L), maintained for three days on shaker at 200 rpm and 28 $^{o}$C. After three days, the cultures were transferred to 20 mL of YM broth with antibiotics and maintained in the same conditions for another day. The absorbance was taken ($\lambda$ = 600 nm) prior to centrifuging at 5000 rpm for 10 minutes. The supernatant was removed and the pellets were re-suspended in antibiotic free bacterial resuspension medium (BRM, Table 1) with appropriate volume to obtain the desired optical densities (0.2, 0.5, 1.0, 1.5) of cells. Acetosyringone was added to BRM at a final concentration of 100 $\mu$M and the culture was shaken at 70 rpm for additional three hours at 25 $^{o}$C.

**Table 6.1:** List of various media used in *Agrobacterium tumefaciens* - mediated transformation of taro embryogenic suspension cells

| Media | Composition |
|---|---|
| YM | 10 g/L mannitol, 0.4 g/L yeast extract, 0.1 mg/L $K_2HPO_4$, 0.4 mg/L $KH_2PO_4$, 0.1 g/L NaCl, pH 6.8 |
| BRM | MS Macro (1/10X), MS Micro (1/10X), MS Vitamins (1X), 85.5 g/L sucrose, 1.0 mg/L thiamine, 0.5 mg/L cysteine, 45 g/L glucose, 100 µM acetosyringone, pH 5.2 |
| BCCM | MS Macro (1/10X), MS Micro (1/10X), MS Vitamins (1X), 50 mg/L myo-inositol, 50 mg/L glutamine, 50 mg/L malt extract, 400 mg/L cysteine, 150 mg/L proline, 1.0 mg/L biotin, 10mg/L ascorbate, 15 g/L sucrose, 15g/L maltose, 5 g/L glucose, 5 g/L PVP, 200 µM acetosyringone, 7 g/L agar, pH 5.3 |
| TCCM | MS Macro (½ X), MS Micro (½ X), MS Vitamins (½ X), 100 mg/L glutamine, 400 mg/L cysteine, 10 mg/L ascorbate, 1.0 mg/L TDZ, 0.5 mg/L 2,4-D, 30 g/L sucrose, 10 g/L glucose, 200 µM acetosyringone, 7 g/L agar, pH 5.3 |
| $CMM_L$ | MS Macro (½ X), MS Micro (½ X), MS Vitamins (½ X), 30 g/L sucrose, 1.0 mg/L TDZ, 0.5 mg/L 2,4-D, 100 mg/L glutamine, pH 5.8 |
| EM | MS Macro (½ X), MS Micro (½ X), MS Vitamins (½ X), 30 g/L sucrose, 0.1 mg/L TDZ, 0.05 mg/L 2,4-D, 100 mg/L glutamine, 7 g/L agar, pH 5.8 |
| GM | MS Macro (½ X), MS Micro (½ X), MS Vitamins (½ X), 0.05 mg/L BAP, 0.1 mg/L IAA, 30 g/L sucrose, 7 g/L agar, pH 5.8 |

### 6.2.5.2 Transformation

Embryogenic suspension cells were harvested four days after subculture and passed through a sieve (500 µm mesh) to remove larger cell clumps. Cells were allowed to settle, SCV adjusted to 1 mL and the supernatant removed. Cells were heat shocked by adding 10 mL of warm (42 $^oC$) $CMM_L$ (Table 1) followed by a further incubation at 42 $^oC$ for five minutes.

The heat-shocked cells were allowed to settle and the supernatant was removed. *Agrobacterium* suspended in BRM was then added to the suspension cells to give a

SCV:BRM ratio of 1:5 (v/v), followed by addition of pluronic acid F68 to a final concentration of 0.02 %. The contents were then centrifuged at 2000 rpm for 10 minutes, followed by a resting phase for 30 minutes at 27 $^{o}$C. Supernatant was removed leaving a SCV:BRM ratio of 1:1 (v/v). The cells were resuspended and approximately 500 µL (SCV) was plated per 70 mm Whatman filter paper placed on co-culture media (BCCM, Table 1) in Petri dishes and incubated in the dark at 22-23 $^{o}$C for three days.

In a separate experiment, the efficacy of BCCM was compared with taro co-cultivation medium (TCCM, Table 1) under the same experimental conditions mentioned above. Further, the effect of acetosyringone concentrations on transformation was investigated by its inclusion in both BRM and TCCM at a final concentration of 0, 100, 200 and 400 µM.

#### 6.2.5.3 Post co-culture treatment and selection

After co-cultivation, the cells were washed three times in $CMM_L$ containing timentin (200 mg/L). After washing, the volume of SCV:$CMM_L$ was adjusted to a ratio of 1:5 (v/v) and then 300 µL aliquots of suspension cells were dispensed onto Whatman filter paper placed on agar-solidified $CMM_L$ containing timentin (200 mg/L) and kanamycin (150 mg/L) and kept in the dark. Cells were transferred onto fresh selection medium every four weeks for two months. They were then transferred to embryogenesis medium (EM, Table 1) containing kanamycin (100 mg/L) and timentin (200 mg/L) and maintained for two more months without subculture for PEM and somatic embryo formation. The PEMs together with the somatic embryos were removed from the filter paper and placed directly on germination medium (GM, Table 1) containing timentin only (200 mg/L).

### 6.2.6 Reporter gene detection and assay

Transient GFP expression was assayed three days post-transformation (for both biolistic and *Agrobacterium*-mediated transformed cells) using a Leica MZ 12 stereomicroscope fitted with a Leica GFP Plus fluorescence module.

Transient β-glucuronidase (GUS) activity was assayed histochemically and fluorometrically as described by Jefferson (1987). Fluorescence was measured using a Perkin Elmer LS50B luminescence spectrophotometer. Values were calculated as GUS activity in ρmoles MU/min/mg protein in transformed cells less the background of non-transformed controls.

### 6.2.7 Confirmation of stable integration of the reporter gene

Since the embryos from both transformation experiments were in germination medium, PCR and Southern blot were not done due to lack of sufficient tissue. However, full characterization will be done once plantlets are established. Therefore, from subsequent microprojectile bombardment and *Agrobacterium* transformation experiments, the number of GFP positive PEMs (green fluorescing PEMs) and SEs were recorded as an indicator of stable transformation.

## *6.3 Results*

### 6.3.1 Kill curve

The effect of various concentrations of kanamycin and hygromycin on growth of non-transformed suspension cells was determined via a kill curve (Figure 6.2A-F). The minimum effective concentration of kanamycin required to inhibit the growth of plated suspension cells was found to be 100 mg/L, with cells showing a gradual necrosis until week 7 by which time all cells had died. The use of 150, 200 and 250 mg/L kanamycin were also effective, although necrosis occurred too rapidly (within two weeks) using 200 and 250 mg/L with all cells necrotic by week 5. To ensure adequate selection, therefore, 150 mg/L kanamycin was chosen as the concentration used for selection of transformed tissue. Hygromycin was too toxic at all the concentrations used (10, 20, 30, 40, 50 mg/L) with all cells turning necrotic within two weeks.

### 6.3.2 Microprojectile bombardment-mediated transformation

#### 6.3.2.1 Transgene expression, selection and regeneration of plants

From five independent bombardment experiments, a total of 120 plates were bombarded with gold microprojectiles carrying the plasmid pART-TEST7 (Figure 6.1). In the first two bombardment experiments, very few cells (average less than 1 per bombarded plate) expressed transient GFP with only two putative transformed embryos forming (one from each cultivar). However, the embryos turned necrotic after three months on hormone-free ½ MS. Therefore, considerable time was devoted towards optimizing the suspension cultures (Chapter 5). As transformation competent suspension cells were established, more plates were bombarded. Transient expression of the *gfp* reporter gene was observed 72 hours post-bombardment with numerous GFP positive (green) foci seen on each bombarded plate of cells (Figure 6.3A & B). No fluorescent foci were observed in non-bombarded cells. The number of green foci observed was considered sufficiently high to attempt selection of stably transformed plants. Therefore, the bombarded cells were transferred to selection medium containing 150 mg/L kanamycin three days after bombardment and maintained in the dark for two months with monthly subculture. After one month on selection medium, untransformed cells began to turn necrotic while putatively transformed cells started to proliferate and form fluorescing callus. The callus was transferred to embryogenesis medium (EM) containing 100 mg/L kanamycin and putatively transformed embryos began to form after 3-4 weeks (Figure 6.4 A & B). Five putatively transformed embryos were obtained from the THA-07 cultivar whereas the GFP positive callus from CPUK became necrotic after two months. The embryos were subsequently transferred to germination (GM) (Figure 6.5) from which kanamycin was omitted due to its suspected interference with embryo germination. However, germination of putatively transformed embryos was very slow. From further bombardment experiments numerous putatively transformed PEMs and SEs were obtained on EM following two months selection (Table 6.2). These embryos are awaiting maturation and germination and will be analyzed once the plantlets are established. No embryos formed from non-bombarded cells on selection.

#### 6.3.2.2 Effect of osmoticum on transgene expression

Osmotic treatment for two hours pre- and post-bombardment significantly increased the frequency of transient GFP expression (Table 6.3) in both cultivars, CPUK and THA-07. The expression level increased by 1.4-fold in suspension cells treated with the osmoticum when compared with the cells without osmotic treatment. In addition, a significant genotypic effect was observed irrespective of osmoticum treatment with THA-07 showing a greater number of fluorescent foci following bombardment.

#### 6.3.2.3 Transient activity of various promoters in taro suspension cells

The activities of different promoters in taro suspension cells was evaluated by fluorometric assay of transient GUS expression from three independent experiments in which there were five replicates for each promoter (Figure 6.6). From the promoters tested, *Ubi*-1 provided the highest level of transient GUS expression followed by CaMV 35S then TaBV-600.

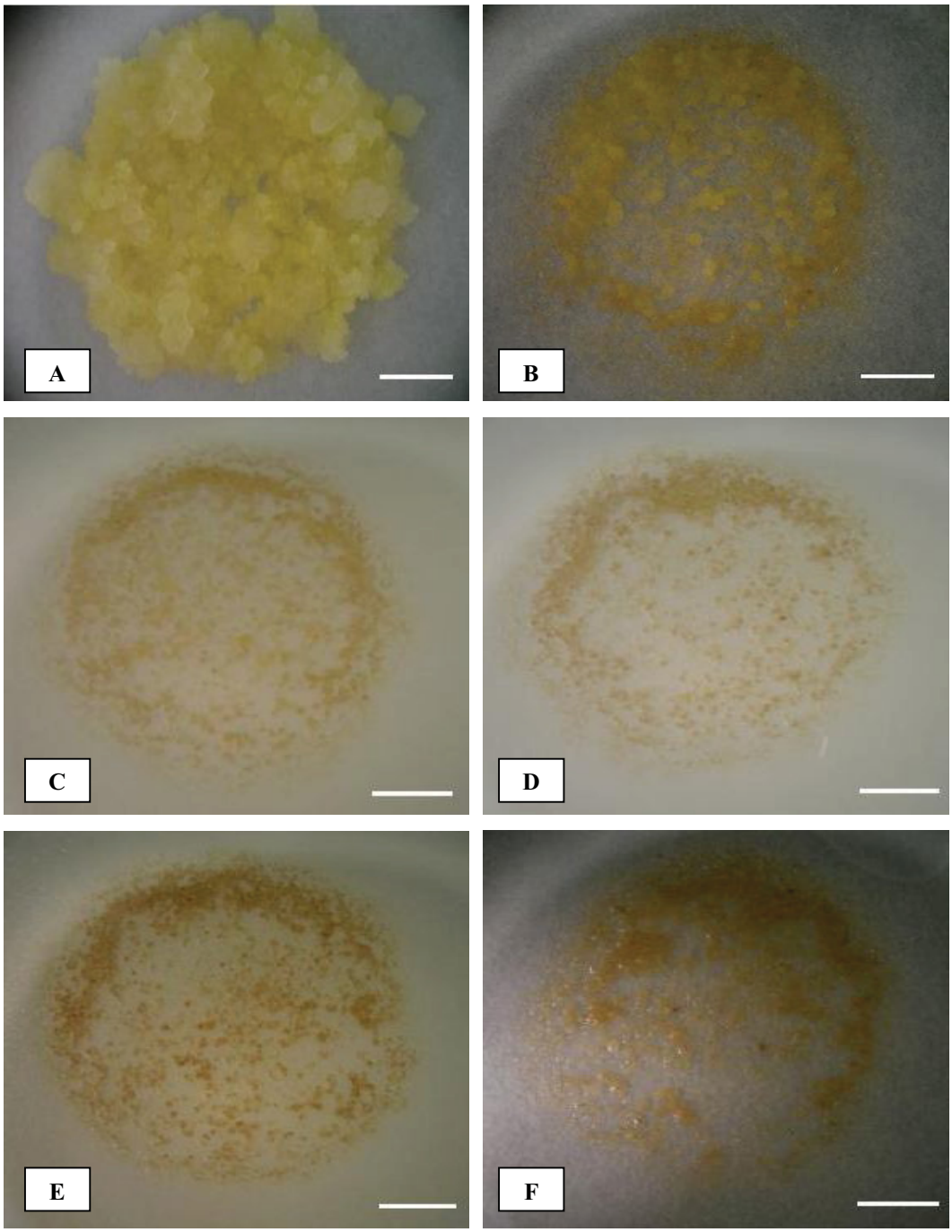


**Figure 6.2:** The effect of kanamycin on the regeneration of non-transformed suspension cells of taro cv. CPUK. Suspension cells were plated onto selection medium containing various concentrations of kanamycin: A = 0 mg/L, B = 50 mg/L, C = 100 mg/L, D = 150 mg/L, E = 200 mg/L and F = 250 mg/L. Picture was taken five weeks after plating. Scale bar = 5 mm.

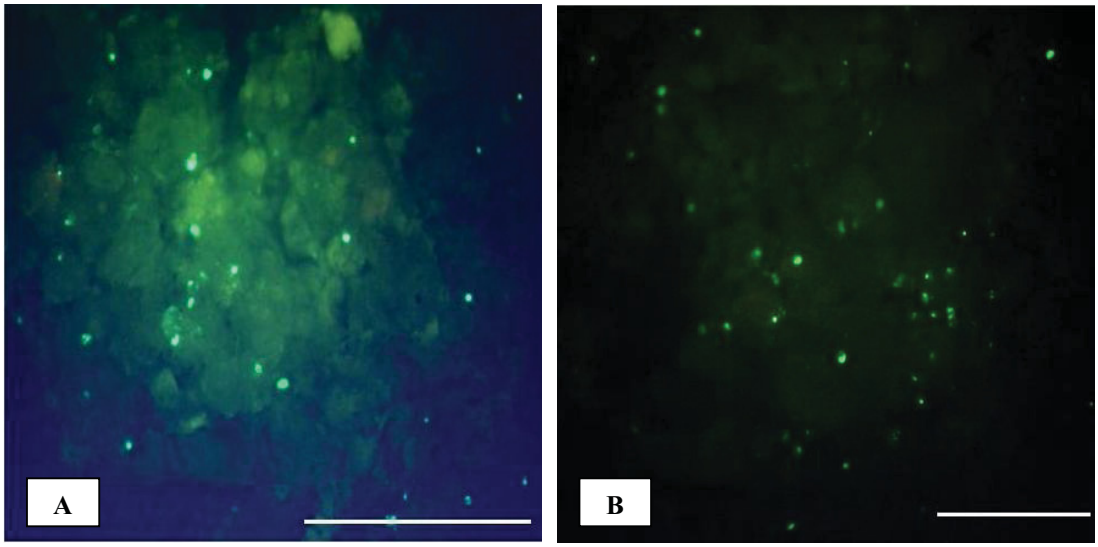


**Figure 6.3:** Transient GFP expression in taro suspension cells three days post-bombardment cv. CPUK (A), THA-07 (B). Scale bar = 1 mm.

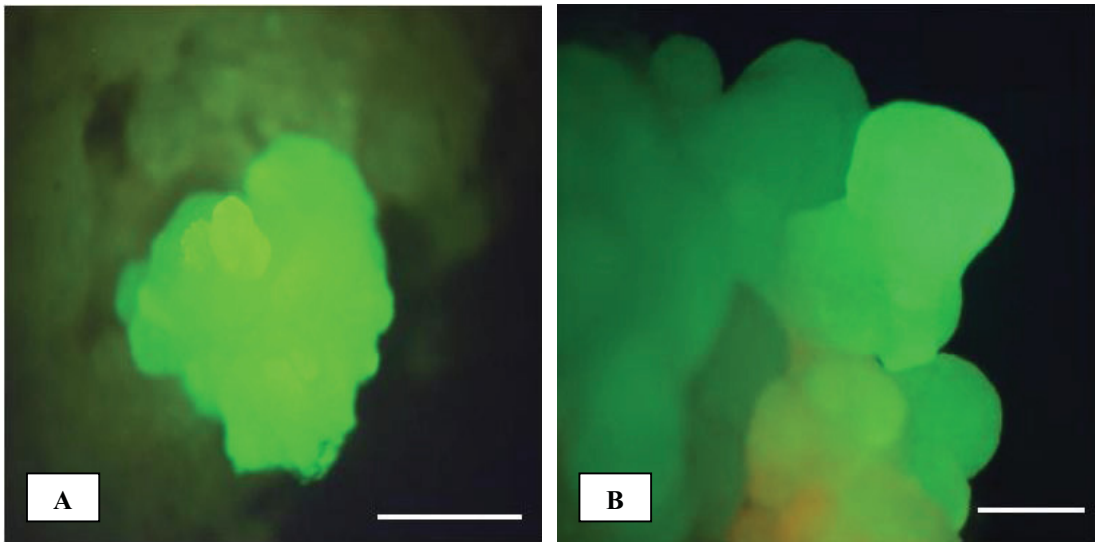


**Figure 6.4:** Selection and regeneration of transgenic taro by microprojectile bombardment. Following transfer onto $CMM_L$ containing 150 mg/L kanamycin, fluorescing, putatively transformed cells multiplied slowly and formed small cell aggregates. Following transfer to EM the transformed cells formed fluorescing PEMs and SEs in both CPUK (A), THA-07 (B) cultivars. Scale bar = 1 mm.

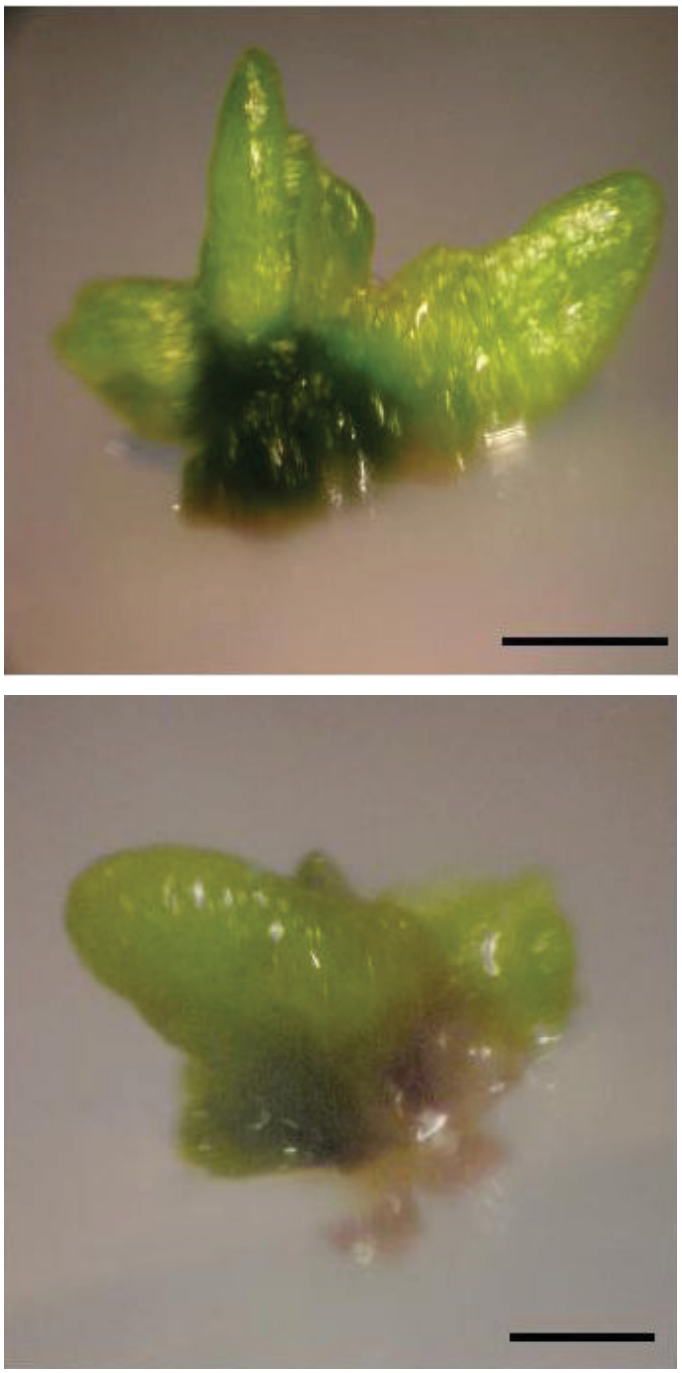

**Figure 6.5:** Putatively transformed taro somatic embryos on germination medium (GM) cv. THA-07. Scale bar = 2 mm

**Table 6.2:** The number of putatively transformed PEMS on embryogenesis medium. After transformation via microprojectile bombardment, suspension cells were kept on selection for two months followed by transfer onto EM.

| Cultivar | Total number of GFP fluorescent PEMs/SEs | Mean number of GFP fluorescent PEMs/SEs per plate | Mean number of GFP fluorescent PEMs/SEs per mL SCV |
|---|---|---|---|
| CPUK | 276 | $14.5 \pm 1.2^{a}$ | 290 |
| THA-07 | 174 | $10.9 \pm 1.5^{a}$ | 218 |

Values with the means ± SEM are derived from 16-19 bombarded plates per cultivar with 50µL SCV per plate. Means followed by the same letter were not significantly different ($p<0.05$).

**Table 6.3:** The effect of osmoticum on transient GFP expression in *Colocasia esculenta* var. *esculenta* three days post-bombardment. Suspension cells were incubated on the osmoticum (0.2 M mannitol + 0.2 M sorbitol) for two hours pre- and post-bombardment and then maintained on $CMM_L$ for three days followed by subculture on selection medium.

| Cultivar | Mean total number of GFP foci per replicate | |
|---|---|---|
| | Osmoticum | Without Osmoticum |
| CPUK | $423 \pm 18.1^{a}$ | $301 \pm 15.2^{b}$ |
| THA-07 | $502 \pm 18.3^{c}$ | $361 \pm 14.3^{d}$ |

Values with the means ± SEM are derived from nine independent bombardment plates for each treatment containing 50 µL of settled cell volume of embryogenic suspension cells. Means followed by same letter were not significantly different ($p<0.05$).

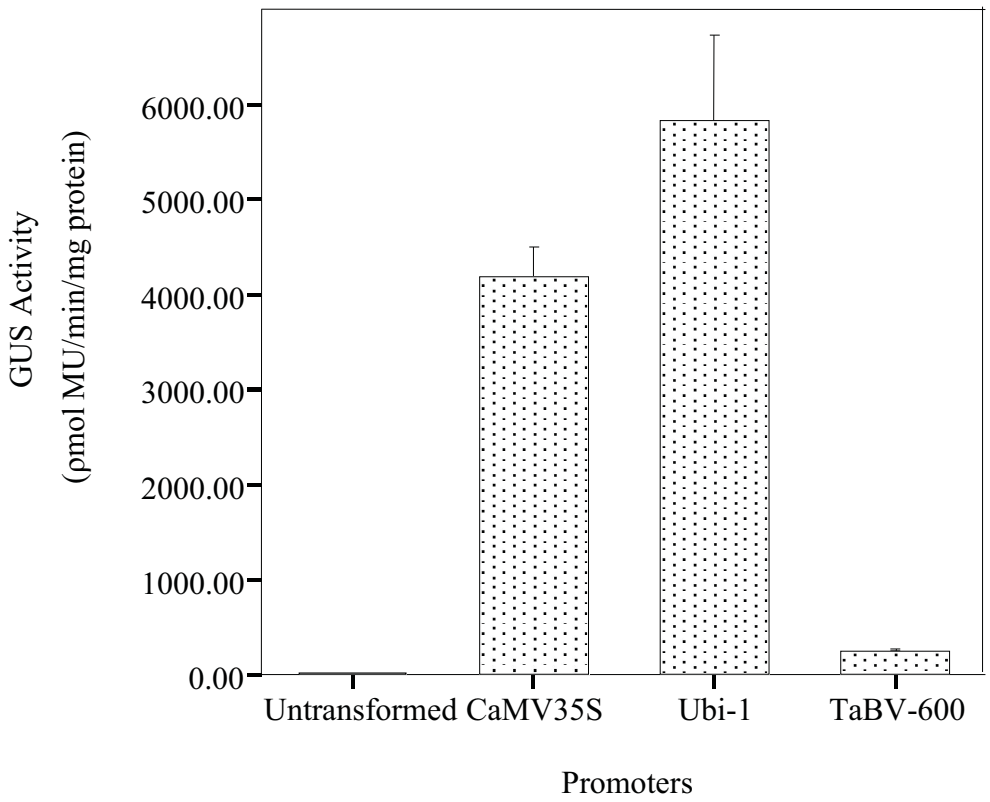


**Figure 6.6:** Comparison of the transient activity of three promoters in taro embryogenic suspension cells using the *uid*A (GUS) reporter gene. Suspension cells were bombarded with expression vectors and promoter activity assayed by GUS fluorometric assay 72 hours post-bombardment. Values are expressed as the mean value ± SEM of three independent experiments with five replicates for each promoter construct per experiment. Values are in ρmol of MU/min/mg total soluble protein. Non-bombarded cells (untransformed) were also included as a control for endogenous GUS activity.

### 6.3.3 *Agrobacterium tumefaciens* - mediated transformation

#### 6.3.3.1 The effect of *Agrobacterium* cell density on transformation

The effect of *Agrobacterium* cell density on transformation efficiency was studied by exposing taro embryogenic cell suspensions to four different concentrations of the bacterium suspended in BRM ($OD_{600}$ = 0.2, 0.5, 1.0, 1.5) (Table 6.4) and comparing the number of fluorescing foci observed between treatments. For cultivar CPUK, the greatest number of GPF foci were observed using an *Agrobacterium* cell density of 0.5 with slightly lower numbers present using ODs of 0.2 and 1. In contrast, an *Agrobacterium* cell density of 1.5 resulted in a significant decrease in the transformation efficiency compared to that obtained using an OD of 0.5. For cultivar THA-07, the greatest number of GFP foci were observed using the lowest *Agrobacterium* cell density tested (OD 0.2). Significantly lower numbers of green foci were observed using an OD of 0.5 with no GFP expression detected in cells exposed to *Agrobacterium* at concentrations of OD 1 and 1.5.

#### 6.3.3.2 The effect of co-culture medium on T-DNA transfer

The effect of the co-cultivation media on transformation efficiency was examined by comparing the transient GFP expression in suspension cells following co-cultivation on either BCCM or TCCM. Co-cultivation media had a significant effect on the transformation efficiency with a more than five-fold increase in the level of transient GFP expression observed in both cultivars when co-cultivated on TCCM in comparison to BCCM (Table 6.5). Transformation efficiency was also significantly different between cultivars irrespective of the co-cultivation media used with approximately double the number of GFP foci observed in THA-07 cells compared to CPUK.

**Table 6.4:** The effect of *Agrobacterium tumefaciens* (AGL1) cell density ($OD_{600nm}$) on transformation efficiency of *C. esculenta* var. *esculenta* embryogenic suspension cells as indicated by the number of GFP positive foci. Embryogenic suspension cells (1 mL SCV) were treated with *Agrobacterium* suspensions (5 mL) at various densities cultured on BCCM for three days in the dark at 23 $^{o}$C then examined for GFP foci.

| Cultivar | *Agrobacterium* cell density ($OD_{600nm}$) | Mean number of GFP foci per replicate |
|---|---|---|
| CPUK | 0.2 | $22 \pm 0.0^{a}$ |
| | 0.5 | $28 \pm 0.5^{a}$ |
| | 1 | $21 \pm 2.5^{ab}$ |
| | 1.5 | $12 \pm 4.0^{b}$ |
| THA-07 | 0.2 | $19 \pm 2.5^{a}$ |
| | 0.5 | $3.0 \pm 1.0^{b}$ |
| | 1 | $0 \pm 0^{b}$ |
| | 1.5 | $0 \pm 0^{b}$ |

Values with the means ± SEM are derived from two replicates of 1 mL of settled cell volume of embryogenic suspension cells. Values followed by the same letter within each cultivar were not significantly different ($p < 0.05$).

**Table 6.5:** The effect of co-cultivation media on transformation efficiency of *C. esculenta* var. *esculenta* as indicated by the number of GFP positive foci. Embryogenic suspension cells (1 mL SCV) were treated with *Agrobacterium* (5 mL; $OD_{600nm}$ = 0.5) then cultured on respective co-culture medium for three days in the dark at 23 $^{o}$C before counting the number of foci.

| Media | Mean number of GFP foci per replicate per cultivar | |
|---|---|---|
| | CPUK | THA-07 |
| TCCM | $118 \pm 2.5^{a}$ | $201 \pm 1.0^{c}$ |
| BCCM | $26 \pm 1.0^{b}$ | $51 \pm 1.5^{d}$ |

Values with the means ± SEM are derived from two replicates of 1 mL of settled cell volume of embryogenic suspension cells. Means followed by the same letter were not significantly different ($p<0.05$).

#### 6.3.3.3 The effect of acetosyringone concentrations on T-DNA transfer

To determine the effect of acetosyringone on transformation, various concentrations (0, 100, 200, 400 μM) were incorporated into both pre-culture (BRM) and co-cultivation (TCCM) media and transient GFP expression levels were compared (Table 6.6). For each treatment, the concentration in both media was kept the same. No GFP expression was observed when acetosyringone was omitted from both media. Further, although the highest number of GFP foci for both cultivars was observed using 100 μM acetosyringone, the differences in GFP expression observed between the different concentrations was not significant.

#### 6.3.3.4 Selection and regeneration

Following co-cultivation, suspension cells were transferred to agar-solidified $CMM_L$ containing 150 mg/L kanamycin and 200 mg/L timentin and observed for transient GFP expression (Figure 6.7A) then maintained in the dark for two months with monthly subculture. Cell necrosis started one week after transfer onto selection and as a result transient GFP expression was reduced to less than 50%. The putatively transformed cells surviving began to proliferate. Following two months selection, these embryogenic clumps formed pro-embryogenic mass on embryogenesis medium (EM) (Figure 6.7 B,C). To avoid possible escapes and to prevent *Agrobacterium* from overgrowing the PEMs, 100 mg/L kanamycin and 200 mg/L timentin, respectively, were included in the EM. From three independent transformation experiments, approximately 17 putatively transformed PEMs per mL of SCV formed from both cultivars (Table 6.7). Molecular characterization will be done once the embryos formed are matured, germinated and established into plantlets.

**Table 6.6:** The effect of different concentrations of acetosyringone on transformation efficiency of *C. esculenta* var. *esculenta* as indicated by the number of GFP positive foci. Embryogenic suspension cells (1 mL SCV) were treated with *Agrobacterium* (5 mL; $OD_{600nm}$ = 0.5) then cultured on TCCM containing different concentrations of acetosyringone for three days in the dark at 23 $^{o}$C before counting the number of foci.

| Concentration of Acetosyringone (μM) | Mean number of GFP positive foci per replicate per cultivar | |
|---|---|---|
| | CPUK | THA-07 |
| 0 | 0 | 0 |
| 100 | $52 \pm 4.5^{a}$ | $181 \pm 9.5^{b}$ |
| 200 | $40 \pm 3.5^{a}$ | $118 \pm 7.5^{b}$ |
| 400 | $20 \pm 3.0^{a}$ | $115 \pm 5.8^{b}$ |

Values with the means ± SEM are derived from two replicates of 1 mL of settled cell volume of embryogenic suspension cells. Means followed by the same letter were not significantly different ($p<0.05$) after natural log transformation.

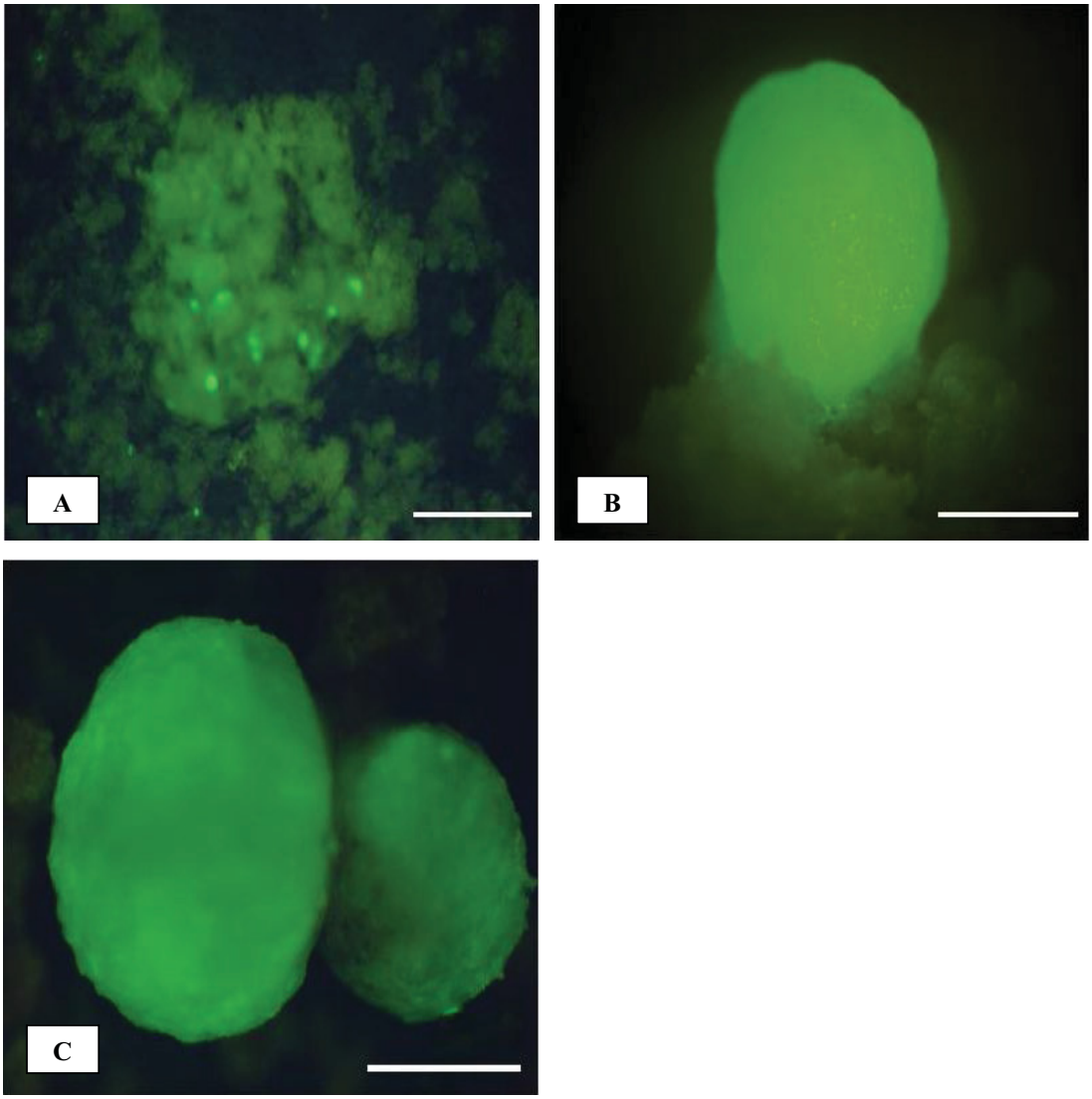


**Figure 6.7:** Taro suspension cells transformed by *Agrobacterium tumefaciens* strain AGL1. Suspension cells (cv THA-07) expressing GFP after three days post co-cultivation (A). After two months on selection, PEMs expressing GFP were observed in both THA-07 (B) and CPUK (C) cultivars. Scale bar = 1 mm.

**Table 6.7:** The number of putatively transformed PEMs on embryogenesis medium. After co-cultivation with *Agrobacterium*, suspension cells were kept on selection for two months followed by transfer onto EM.

| Cultivar | Total number of fluorescent PEMs | Mean number of PEMs expressing GFP per mL SCV |
|---|---|---|
| CPUK | 87 | 15 |
| THA-07 | 19 | 19 |

Fluorescing PEMs were counted after one month following transfer onto EM. Total for CPUK is from 6 mL SCV and for THA-07 is from 1 mL SCV.

## *6.4 Discussion*

The ability to select transformed plant tissue from non-transformed tissue is an important pre-requisite in the generation of transgenic plants. The most commonly used selectable marker genes in plant transformation are antibiotic and herbicide resistance genes. At the commencement of this study there was very little information on suitable selective agents for the generation of transgenic taro. In one report using *C. esculenta* var. *antiquorum* callus tissue growing on solid media, tissue was found to survive on 200 mg/L kanamycin, while all tissue became necrotic on 20 mg/L hygromycin (Fukino *et al.*, 2000). In another study, He *et al* (2008) used geneticin (50 mg/L) for selection of transformed callus in *C. esculenta* var. *esculenta*, but reported there were some escapes. An interesting outcome from these studies was that, despite the use of kanamycin and geneticin at concentrations generally regarded as high (200 and 50 mg/L, respectively), they were not effective in preventing the growth of non-transformed tissue.

Based on the results from these previous studies, the effectiveness of kanamycin and hygromycin as selective agents was further examined. The requirement for effective selection is the gradual cessation of growth of non-transformed cells rather than rapid death. The minimum effective concentration of kanamycin preventing the growth of non-transformed cells was found to be 100 mg/L. At 200 and 250 mg/L kanamycin, cells were killed too rapidly. This is considered undesirable as dead and dying cells may release substances deleterious to neighboring transgenic cells and reduce transformation efficiency. To reduce the risk of escapes, a kanamycin concentration of 150 mg/L was chosen rather than 100 mg/L. The use of hygromycin at the four concentrations tested was considered unsuitable for selection since even the lowest concentration (10 mg/L) resulted in rapid cell death. The effectiveness of lower concentrations of hygromycin was not examined. The differences in the results obtained in this and previously reported studies may have been genotypic differences. However it is more likely that the differences are related to the type of tissue used; cells plated from suspension cultures form a thin layer where a higher proportion of cells are closer to the medium in comparison to callus derived from solid culture.

Initially, attempts were made to transform taro embryogenic suspension cells by microprojectile bombardment using the parameters described for transforming banana cell suspensions (Becker, 1999). Since these parameters resulted in high transient GFP expression (more than 100 GFP foci per plate) they were used in all subsequent transformation experiments. The only variation was that the time between plating the cells and bombardment was extended from four days to six days since this resulted in a higher transformation efficiency (data not shown). Similar results were obtained by O'Kennedy *et al* (2001) and Patnaik and Khurana (2003) in white maize and wheat, respectively. It is likely that the culturing of taro suspension cells on agar-solidified $CMM_L$ for six days prior to shooting allowed the cells to adapt to the altered growing conditions and begin to proliferate thus increasing the number of actively dividing competent cells. Such actively dividing cells are reported to be ideal target tissue for particle bombardment as (i) they can easily overcome the stresses induced by microprojectiles (Santos *et al.*, 2002) and (ii) high frequency of transgene integration into the host genome, thus increasing the probability of obtaining stable transgenic plants (Yang *et al.*, 1999).

The addition of an osmoticum treatment both pre- and post-bombardment resulted in a 1.4-fold increase in the frequency of transient GFP expression. A similar effect has also been reported in a number of plants such as *Picea abies* (Clapham *et al.*, 1995), asparagus (Cabrera-Ponce *et al.*, 1997), oil palm (Parveez *et al.*, 1998), potato (Romano *et al.*, 2001), wheat (Rasco-Gaunt *et al.*, 2001), maize (Rasco-Gaunt *et al.*, 2001), blue grama grass (*Bouteloua gracilis*) (Aguado-Santacruz *et al.*, 2002), garlic (Sawahel, 2002) and Bermuda grass (Li and Qu, 2004). Osmotic treatment reduces turgor pressure thus reducing cell damage, particularly, leakage of protoplasm (Clapham *et al.*, 1995; Marchant *et al.*, 1998; Yang *et al.*, 2001; Santos *et al.*, 2002). Although increasing transformation efficiency in many cases, high transgene expression can still be obtained in some species, such as banana suspensions, without osmotic treatment (Becker, 1999). It could be that cells of these species are able to recover from the stress induced by bombardment without difficulty.

Selection of transformed cells was initially done by culturing bombarded cells on solidified $CMM_L$ containing 150 mg/L kanamycin for two months. By end of the second month, the non-transformed cells were necrotic. Fluorescing PEMs and SEs began to form four weeks after transfer of the cells on EM. In order to promote embryogenesis but still maintain some selection, the concentration of kanamycin was reduced to 100 mg/L. Kanamycin was omitted from the germination medium because it appeared to be inhibitory to embryo conversion as green fluorescent embryos that formed on selection were extremely slow to germinate. The presence of strongly fluorescing cells coupled with the fact that no embryos formed in the non-transformed control cells on selection strongly indicates that the embryos were not escapes. The inhibitory effect of kanamycin has been reported in other monocotyledons such as *Alstroemeria* (van Schaik *et al.*, 2000) and *Panicum maximum* (Kothari *et al.*, 2005). Therefore, a different selection regime may aid in regenerating transgenic plants without the need to remove selection at the germination step. Such selection may be a different antibiotic, a herbicide or positive selection.

Transient analysis of promoter activity in taro cell suspensions was done by fluorometric assays of GUS expression. These studies showed that the maize polyubiquitin-1 (*Ubi*-1) and cauliflower mosaic virus 35S (CaMV 35S) promoters were more active in this cell type than the taro bacilliform virus (TaBV-600) promoter. The level of *Ubi*-1 activity was 1.4 and 23-fold higher than the CaMV 35S and TaBV-600 promoters, respectively, while that of CaMV 35S was 16-fold higher than TaBV-600. Interestingly, using transient assays in detached taro leaves, Yang *et al* (2003) reported that the activity of the TaBV-600 promoter was greater than CaMV 35S. This indicates that TaBV-600 promoter may be more active in differentiated rather than undifferentiated cells of taro. A comparison of promoters in stably transformed taro is required to fully assess and compare the strength and tissue specificity of these promoters.

In addition to microprojectile bombardment, the use of *Agrobacterium* to transform embryogenic cell suspensions of *Colocasia esculent* var. *esculenta* was also investigated. Initially, various factors known to affect the efficiency of *Agrobacterium*

*tumefaciens*-mediated transformation were examined including bacterial cell density, co-cultivation medium and concentration of acetosyringone. *Agrobacterium* density did not appear to have a significant impact on transformation efficiency until a threshold was exceeded. However, this was difficult to determine for cultivar THA-07 as the highest transformation frequency occurred at the lowest density. In preliminary experiments an $OD_{600}$ = 0.5-0.7 resulted in higher transformation efficiency in cultivar THA-07. This discrepancy was probably due to the transformation competency of the cell line at the time as in later experiments using an OD of 0.5, the transformation efficiency of THA-07 was greater than that of cultivar CPUK. A decrease in transformation efficiency with increased concentrations of *Agrobacterium* is most likely the result of cell death due to high bacterial numbers (Carvalho *et al.*, 2004) rather than poor T-DNA transfer. This phenomenon has been observed in banana suspension cells (Khanna *et al.*, 2004). In fact cell necrosis was observed in taro suspension cells two weeks after co-cultivation, with greater cell death associated with higher bacterial densities (OD 1, 1.5)

The *Agrobacterium*-mediated transformation protocol used for taro was based on that currently used at Queensland University of Technology for banana (Khanna *et al.*, 2004). As such, the co-cultivation media described for banana (BCCM) was initially used. This media was based on the nutritional and hormonal requirements of banana and may not have been optimal for taro. To improve the efficiency of transformation, a taro co-cultivation medium (TCCM) was formulated which consisted $CMM_L$ plus 10 g/L glucose, 400 mg/L cysteine, 10 mg/L ascorbate, 200 μM acetosyringone, pH 5.3. A dramatic increase (four-fold) in transient GFP expression was obtained in both taro cultivars on TCCM when compared with the level of expression on BCCM. This increase in transformation efficiency may have been due to more optimal nutrient and hormone concentrations or simply less shock from the change to a different nutritional regime. In support of this latter theory, cells co-cultivated on BCCM turned brown and showed signs of stress and necrosis on the third day of co-cultivation whereas this did not occur using TCCM.

A significant difference in the transformation efficiency was observed between the two taro cultivars used in this study. Genotypic differences in transformation competence have been well documented in other plant species such as *indicia* rice cultivars HKR-46 and HKR-126 (Saharan *et al.*, 2004) and wheat (Bhalla *et al.*, 2006). The variable responses are due to a biological interaction between the bacterial and plant cells (Khanna *et al.*, 2004) and may be overcome by the use of different *Agrobacterium* strains (de la Riva *et al.*, 1998; Chakrabarty *et al.*, 2002; Mohamed *et al.*, 2004) or modulation of the plant response such as inhibiting the programmed cell death response (Khanna *et al.*, 2007). A more difficult problem to overcome would be a deficiency in host genes required for T-DNA transfer and integration (Zhu *et al.*, 2003).

Acetosyringone has been reported to be critical for transformation in many monocotyledons such as wheat (Cheng *et al.*, 1997), banana (Khanna *et al.*, 2004), rice (Nishimura *et al.*, 2006), barley (Shrawat *et al.*, 2007), and in dicotyledons such as soybean (Dang and Wei, 2007). This phenolic compound is secreted from wounded plant cells and induces the *Agrobacterium vir* genes thus initiating T-DNA transfer and transformation (Suzuki *et al.*, 2001). The removal of acetosyringone from co-cultivation medium in this study resulted in a lack of GFP expression in taro suspension cells. This result suggested that like other monocots, taro cells do not produce *vir*-inducing phenolic compounds such as acetosyringone. In general, increasing the concentration of acetosyringone from 100 μM to 400 μM decreased the frequency of transformation although the difference was not significant. A reduction in the frequency of transformation with higher concentrations of acetosyringone has also been reported in rice (Saharan *et al.*, 2004), barley (Kumlehn *et al.*, 2006) and orchard grass (Lee *et al.*, 2006). Higher concentrations of acetosyringone also decreased the growth of *Agrobacterium* co-cultivated with androgenetic pollen grains of barley, resulting in a very low transformation (Kumlehn *et al.*, 2006). The concentration at which acetosyringone is most effective is variable and appears to be dependent on the *Agrobacterium* strain and plant genotype. Since acetosyringone is effective at low concentrations and becomes bacteriostatic or bactericidal at higher concentrations, the effective concentration should be determined for different strains

of *Agrobacterium* used in transformation. An alternative is the use of superbinary vectors where the vir G is constitutively expressed removing the requirement for acetosyringone (de la Riva *et al.*, 1998).

The frequency of transformation in taro embryogenic suspension cells was higher via microprojectile bombardment than *Agrobacterium*-mediated transformation in the present study. However, it cannot be ascertained which transformation system is better, unless the putatively stable transgenic plants are characterized. In this study, both systems gave acceptable results; hence both systems can be used for transformation studies in taro. He *et al* (2004) reported very low transformation efficiency (only 1 stable transgenic plant) via microprojectile bombardment. In this study five stably transformed germinating embryos were obtained from THA-07 cultivar and numerous putatively transformed PEMs and somatic embryos from both cultivars. These will be characterized once grown to sufficient size. Similarly, many putatively transformed PEMs have been obtained via *Agrobacterium* which is also indicative of high transformation. Therefore, based on these results, it could be said that microprojectile bombardment gave a higher transformation frequency than *Agrobacterium*-mediated transformation in *C. esculenta* var. *esculenta*. Further, this study also showed that high frequency of transformation in taro could be achieved when suspension cells are used as the target tissue. The low frequency of transformation in *C. esculenta* var. *esculenta* via microprojectile and *Agrobacterium* in previous studies (He *et al.*, 2004; 2008) could have been due to the use of callus as target tissue. Further, heat shocked cells were used for *Agrobacterium*-mediated transformation in this study unlike the previous research. One of the factors which appeared to hinder the fast recovery of putatively stably transformed plantlets in the present study was kanamycin. The previous research group (He *et al.*, 2004; 2008) used geneticin as the selective agent. The effect of geneticin on the regeneration process is unknown but the use of this selective agent reportedly produced ‘escapes’.

This study has described a preliminary investigation into the genetic transformation of *Colocasia esculenta* var. *esculenta* by both *Agrobacterium* and microprojectile bombardment–mediated methods. Putative stable transgenic proembryos and embryos

expressing GFP were successfully produced by both methods are awaiting maturation, germination, plantlet development and molecular characterization.

# CHAPTER 7

## General Discussion and Conclusions

Regeneration and transformation systems are indispensable modern plant breeding components since they provide an alternative platform to develop control strategies against the plethora of pests and diseases affecting many agronomic crops. Prior to this study, there were no reports on indirect somatic embryogenesis either via an intervening callus or suspension cells in *Colocasia esculenta* var. *esculenta*, the preferred taro in the Pacific. Further, there were only two previous reports of stable transformation of the *esculenta* variety. He *et al* (2004) transformed the Chinese taro cultivar Bung long by microprojectile bombardment of callus and obtained one stable transformant. He *et al* (2008) also transformed the same taro cultivar using the same target tissue by *Agrobacterium tumefaciens* and obtained six stable transformants. While these studies show that both transformation methods could be used to introduce foreign genes into taro, the transformation efficiency appeared to be very low. Such low transformation efficiency is undesirable, since many transformants need to be generated in order to generate transgenic plants exhibiting the desired trait while maintaining the same agronomic properties of the parent cultivar. As taro is vegetatively reproduced, there would be no need to backcross transgenes into elite adopted germplasm. Therefore, this study was undertaken to develop an efficient indirect somatic embryogenesis system for *C. esculenta* var. *esculenta* to enable large-scale propagation and to provide a large amount of suitable target tissue for transformation. Therefore, various *in vitro* parameters were studied systematically in order to optimize the initiation of embryogenic callus, proliferate this material and to regenerate plants. Twelve taro genotypes were initially available for use in this study. However, in preliminary screening for the presence of endogenous bacteria, only three genotypes, CPUK, CK-07 and THA-07, tested negative and could be used in further study.

The requirement for exogenous growth regulators in somatic embryogenesis is well known. It is also clear, however, that the type and concentration of growth regulator varies between species or even cultivars and between explants within the same species. The plant growth regulators 2,4-D and TDZ were selected for this study since previous research has demonstrated their role in somatic embryogenesis in some monocots (Thinh, 1997; Srangsam and Kanchanapoom, 2003; Gairi and Rashid, 2004; Verma *et al.*, 2004). Furthermore, these growth regulators have a relatively high activity in comparison to other plant growth regulators (Thinh, 1997; Bhuiyan and Adachi, 2002; Lin *et al.*, 2004; Shinoyama *et al.*, 2004) and thus had the potential to overcome the recalcitrance to callus formation reported in the *esculenta* taro variety (Jackson *et al.*, 1977).

In this study two combinations of 2,4-D (1.0, 2.0 mg/L) and TDZ (0.5, 1.0 mg/L) were found to be effective at initiating regenerable callus corm explants with a 2,4-D and TDZ combination of 2.0 mg/L and 1.0 mg/L, respectively, the most effective. This was demonstrated in three genotypes, CPUK, CK-07 and THA-07. It remains to be seen whether other genotypes of the *esculenta* variety respond in a similar manner or if some genotypes prove completely recalcitrant.

Murashige and Skoog (1962) medium was selected on the basis of previous studies which showed that taro responded satisfactorily to callus initiation when cultured on this medium (Yam *et al.*, 1990; Yam *et al.*, 1991; He *et al.*, 2004). Half-strength MS was found to be more effective than full or quarter-strength media. It is possible that alternative media containing similar levels of inorganic salts may be more suitable and that further refinements could be made. When embryogenesis is used for transformation and a satisfactory number of transformants are generated, it may be questionable whether further refinement is warranted. However, since maximum efficiency is required to make a micropropagation system commercially viable, further study into such refinements should not be considered trivial.

Despite using various explants such as shoot-tips, root-tips, mid-rib, petioles and leaf primordial, only corm sections formed regenerable callus. The corms slices used as

explants were derived from plants grown on hormone-free media for 12 to 16 weeks. Juvenile tissues are considered preferable for somatic embryogenesis since they contain a higher proportion of meristematic cells (Lakshmanan and Taji, 2000; Dhar and Joshi, 2005) thus can be stimulated to undergo embryogenesis. As taro is vegetatively propagated, the use of the terms "juvenility" may not deemed appropriate as it may be many years, possibly hundreds, since some cultivars had germinated from seed. However, it may be that other factors affect the embryogenic response such as time since subculture or the position of the slice within the corm or the size of the corm. Corms are the sites where axillary buds are present and larger corms produce more axillary buds, an observation noted during the course of this study. It is most likely that the meristems in axillary buds are sites where regenerable callus originates. Whether there are more meristems in this part of the corm or whether actively growing plants have a better embryogenic response requires further experimentation. The effect of the age of corm explants or the position of the slice within the corm on the frequency of somatic embryogenesis may also warrant further investigation.

High frequency callus induction and growth have been correlated to endogenous calcium in plant tissue explants. For example, increasing calcium levels in *Chrysanthemum monifolium* increased callus induction significantly (Borgatto *et al.*, 2002). Calcium functions as a secondary messenger in intracellular signaling and may play a role in embryogenic processes (Borgatto *et al.*, 2002). Since taro corms contain substantial amounts of endogenous calcium (~34 mg/100 g tissue) (Bradbury and Holloway, 1988), it is possible that endogenous calcium may have enhanced embryogenic callus formation in taro corm slices or that higher calcium concentration may give an increased response in corm slices or other explants.

The initiation of regenerable callus from the taro corm explants in this research took 3-4 months. Such a long initiation period has also been reported in *Alocasia* (Thao *et al.*, 2003), a closely related genus to *Colocasia* and appears to be common in other non-graminaceous monocots. Further studies aimed at reducing this time period may make a significant contribution in optimizing taro somatic embryogenesis protocol.

However, once cultures are established, this long lead-time may not be a significant limitation with the proviso that cultures can be used for long periods and the frequency of somaclonal variation does not increase over acceptable limits.

Despite a lengthy initiation time, the embryogenic callus still multiplied and substantial amounts were generated in a relatively short time frame, both in solid and liquid media. Callus on solid maintenance medium maintained its regenerabilty for almost two years compared with at least ten months for suspension cultures. This provided a window of approximately 6-7 months in which to use suspension cultures for transformation and regeneration experiments. Therefore, fresh suspensions were initiated after six months from the existing callus while new callus was initiated after one year. Fresh initiations not only ensured a continuous supply of regenerable embryogenic material but also minimized the incidence of somaclonal variation.

During the proliferation of callus on solid media, sectors of non-embryogenic callus were formed. Although these could be removed manually, it is possible that their production could be prevented by the addition of sorbitol since this has been shown to prevent the formation of non-embryogenic callus in maize (Swedlund and Locy, 1993). The effect of sorbitol on callus proliferation was not examined in this study, however, the development of such a callus maintenance medium would reduce the labor intensive and tedious process of selecting for embryogenic callus during subcultures.

Considerable experimentation was dedicated to optimizing protocols for initiation, maintenance and regeneration from suspension cultures. Breaking callus clumps manually with a pipette during subculture facilitated the release and formation of single and multicellular aggregates. However, cell viability and regenerability was a major issue when cells were transferred to regeneration media since a high proportion of cells became necrotic. Difficulties were also experienced with inducing viable cells to produce embryos, but these were overcome by trialing various media formulation. Despite successfully initiating large number of embryos, development was frequently arrested at globular stage resulting in an embryo germination frequency of

approximately 41 %. Consequently, factors facilitating embryo development, such as the use of PEG-4000, other carbohydrates, GA, and ABA, require further investigation so that the full potential of somatic embryogenesis as a mass propagation system for taro is realized.

Despite no phenotypic aberrations observed in any of the acclimatized plants generated in this study, the possibility of somaclonal variation cannot be dismissed. Extensive field trials using large numbers of plants will be required to ascertain whether somaclonal variation occurs, to characterize aberrant phenotypes and to observe how frequencies change over culture time. In addition, the vigor of plants generated by this method, in terms of their growth rate, pest and disease resistance and corm characteristics including size, quality and taste also needs to be investigated. Interestingly, banana plants derived from tissue culture are more vigorous than field-derived suckers but are more susceptible to pests and diseases (Smith *et al.*, 1998). Finally, the cost and feasibility of large-scale plant production using embryogenesis needs to be evaluated to ascertain whether this is commercially viable.

One means of minimizing somaclonal variation is cryopreservation (storage in liquid nitrogen). Once established, callus could be stored until the existing cell lines exceed the culture time and multiplication rate considered safe to keep somaclonal variation below the desired threshold. A cryopreservation protocol has been developed for *C. esculenta* var. *esculenta* shoot-tips (Sant *et al.*, 2008), but there are no reports on cryopreservation of embryogenic callus. The major challenges in the development of a cryopreservation protocol for taro embryogenic callus would be the determination of: (i) the minimum effective size of embryogenic callus that could be frozen without killing the meristematic cells, (ii) the embryogenic potential of the callus after thawing from liquid nitrogen and (iii) the effective means for desiccating the callus to the required moisture content so that freezing could be done without ice formation. Cryopreservation of embryogenic callus or somatic embryos could also be another means of taro germplasm conservation.

A further step along the path of using embryogenesis for mass propagation involves synthetic seeds. Synthetic seeds are somatic embryos encased in a protective coating like alginate hydrogel. When planted in a suitable medium, the coating decomposes and the somatic embryo germinates like a normal seed (Saiprasad, 2001). This would eliminate the labour costs and space requirements for embryos germinated *in vitro* and may provide a means of storing and moving germplasm. There are contrasting reports on the dormancy and viability of taro seeds produced by sexual hybridization (Strauss *et al.*, 1979; Wang, 1983) and this may also be problematic for somatic embryos.

Embryogenic suspension cells have been reported to be the preferred target material for genetic transformation due to (i) their unicellular origin resulting in non-chimeric transgenic plants, (ii) the relatively high surface area of small cell clumps providing maximum exposure to the transforming agent and selective agent and (iii) the high frequency of transgene integration in actively dividing cells (Mahn *et al.*, 1995). Both *Agrobacterium tumefaciens* and microprojectile bombardment-mediated transformation were used to generate putative stably transformed embryos expressing the *gfp* reporter gene. Molecular analysis of plants to confirm transgene integration could not be done, as embryos had not yet developed into plantlets to provide sufficient tissue samples. These germinating embryos will be characterized once they are fully developed into individual plantlets, which could take 8-10 months. However, the continued expression of the GFP by the embryos after six months on selection (kanamycin 100 mg/L) is a strong indicator that the reporter and marker genes are stably integrated into the taro genome. Importantly, no green fluorescence or advanced embryo development was observed in non-transformed control cultures. Comparison studies on whether the transgenic taros are phenotypically aberrant from their wild counter parts need to be done also as it would be valuable in taro transgenesis.

Even though a high frequency of putative transgenic embryos was obtained using biolistics as opposed to *Agrobacterium*, the latter system is still preferable as it is considerably less expensive (Lee *et al.*, 2006), results in a lower transgene copy number and can transfer large segments of DNA with less transgene rearrangements

(Jones, 2005) thus reducing the probability of gene silencing. Which system provides greater freedom to operate is dependent on the country where transformation is undertaken, where the crop is to be grown and, if the crop is exported, the destination and form in which it is exported. This is a complex question and can only really be answered by a thorough intellectual property audit.

Although it is now possible to confer many different traits through transgenesis, the highest priorities for taro would be resistance to taro leaf blight and taro beetle, which are considered the most serious threats to production. Partial resistance to some fungal diseases has been generated in some crops transformed with the chitinase gene derived from plants such as rice. Chitinase hydrolyses the carbohydrate components of fungal cell walls, namely chitin and β-1,3-glucan (Broglie and Broglie, 1993). Other approaches include antimicrobial proteins (Tripathi *et al.*, 2004) and anti-apoptotic genes (Dickman *et al.*, 2001). Such an approach may be useful in generating resistance to taro leaf blight. Identifying a suitable promoter driving these genes in taro leaves may be an issue requiring considerable experimentation on gene expression pattern and stability in transgenic taro. Although a preliminary promoter study was undertaken in this present research, a greater range of promoters in stably transformed plants in the field need to be assessed.

There are also challenges for generating transgenic taro resistant to taro beetle. A protein toxic to taro beetle but innocuous to humans and livestock would be required as well as a promoter which could drive the appropriate level of expression in corms. One potential class of proteins are protease inhibitors such as trypsin inhibitors, which are also derived from plants. These have been reported to confer resistance against pests in some crops such as tomato, potato, rice, strawberry and tobacco. These inhibitors control the growth of insect larvae by inhibiting their gut proteases (Mochizuki *et al.*, 1999; Bell *et al.*, 2001; Shukla *et al.*, 2005). Pusztai *et al* (1992) reported that the cowpea trypsin inhibitor (CpTI) does not have serious long-term effects on the nutritional value of food. This gene, being of plant origin, would be of less concern to the public than genes derived from other organisms.

The introduction of biotechnology to improve food crops in Pacific Island countries will take some time. There is no legislation on the use of biotechnology and genetically modified food crops in many Pacific Island countries. Although this technically means there is no regulatory restriction, transgenic crops must be introduced in consultation with government and the public. Some of the Pacific Island countries have drafted biosafety frameworks under the United Nations Environment Programme (UNEP)-Global Environment Facility (GEF) as a precautionary measure (UNEP-GEF, 2008). However, many of these countries are open to the benefits that modern biotechnology could provide. Further, with the considerable US influence in the Northern Pacific, it is likely that agricultural biotechnology will soon enter this region. Indeed, GM papaya is now commercially cultivated in Hawaii (Gonsalves and Ferreira, 2003).

In this study, efficient somatic embryogenesis and transformation systems for taro were developed. Both of these systems have the potential to significantly contribute to the mass production of disease-free planting material and to the molecular breeding of taro which, to date, has been neglected. Although further refinements of these systems can be made, they are already sufficiently developed for the technology to be applied in addressing some of the limitations to taro production.

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