In Vitro Plant Regeneration in *Catharanthus Roseus (L.) G. Don*

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxy acetic
ABA	Abscisic acid
BA	6-benzyladenine
BAP	6-benzylaminopurine
B5	Gamborg B5 medium
GA_3	Gibberellic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog's (1962) medium
α-NAA	α-Naphthalene acetic acid
PCR	Pacifica Cherry Red
P-Dp	Purple petal, Dark purple centre
PGR	Plant growth regulator
SEs	Somatic embryos
TDZ	Thiadiazuron
W-Y	White petal, Yellow centre

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

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ABSTRACT

Catharanthus roseus is an important medicinal plant that contains two well known anticancerous alkaloids, vincristine and vinblastine. Three factors were found to be important for *in vitro* plant regeneration: (1) the type of explants, (2) the concentrations and type of plant growth regulators, and (3) the genotype. In this study, mature embryo and stem node explants were induced to develop multiple shoots by culturing them on basal medium (MS salts and B5 vitamins) supplemented with TDZ. For stem node explants of *C. roseus* cv. P-Dp, the highest shoots multiplication rate was obtained in medium supplemented with 2.0 mg/L TDZ. While among mature embryo explants of the four cultivars of *C. roseus*, only cv. Pink and cv. Pacifica Cherry Red formed multiple shoots in medium supplemented with 3.0 mg/L TDZ and 2.5 mg/L TDZ, respectively. Mature embryo of cv. W-Y and cv. P-Dp gave poor response and did not form multiple shoots in all treatments. The regenerated plantlets from stem node explants were easily rooted on basal medium devoid of plant growth regulators. The rooted plantlets were acclimatized and planted out in the plastic cups. Apart from plant regeneration through shoots proliferation, embryogenic callus in *Catharanthus roseus* cv. P-Dp was initiated from hypocotyl on basal medium supplemented with 2,4-D. Somatic embryos were successfully induced from primary callus on basal medium supplemented with NAA after three months of culture but the frequency is not high. Petiole and leaf explants of *C. roseus* cv. P-Dp showed highest callusing rates (100%) in the presence of TDZ, however, after three months of culture, no somatic embryo was observed from the callus in both types of explants.

Key words: *Catharanthus roseus*, mature embryo, hypocotyl, stem node, organogenesis, somatic embryo

ABSTRAK

Catharanthus roseus merupakan sejenis tumbuhan perubatan yang terkenal dengan kandungan anticancer alkaloid, vincristine dan vinblastine. Tiga faktor ditemui penting bagi regenerasi in vitro: (1) jenis eksplan, (2) kepekatan dan jenis zat pengatur tumbuh, dan (3) genotip. Melalui kajian ini, eksplan embrio matang dan node batang diinduksikan untuk menghasilkan pucuk-pucuk baru apabila dikulturkan ke medium dasar (garam MS dan vitamin B5) yang mengandungi TDZ. Bagi eksplan node batang dari C.roseus cv. P-Dp, kadar tinggi dalam penghasilan pucuk baru didapati pada media mengandungi 2.0 mg/L TDZ. Manakala embrio eksplan dari empat jenis variasi C. roseus, hanya cv. Pink dan cv. Pacifica Cherry Red regenerasi dengan efisien melalui organogenesis dalam media mengandungi 3.0 mg/L TDZ dan 2.5 mg/L TDZ masing - masing. Embrio matang dari cv. W-Y dan cv. P-Dp memberi respon yang rendah dan tidak manghasilkan pucuk-puncuk baru dalam semua eksperimen. Pucuk dari eksplan node batang berakar dalam media dasar tanpa zat pengatur tumbuh. Pucuk berakar disesuaikan dan ditanamkan dalam bekas plastik. Selain daripada regenerasi melalui penghasilan pucuk, kalus embrio dapat diinduksikan dengan menggunakan eksplan hipokotil cv. P-Dp dalam media mangandungi 2,4-D. Somatik embrio berjaya berbentuk pada kalus dalam dasar media mengandungi NAA selepas dikultur tiga bulan. Kadar pembentukan kalus yang baik bagi eksplan daun dan tangkai daun adalah dengan kehadiran TDZ, dimana sebanyak 100% kalus dihasilkan bagi kedua-dua jenis eksplan. Walaubagaimanapun, selepas pengkulturan tiga bulan, somatik embrio tidak dapat diinduksikan melalui kalus dari daun dan tangkai daun eksplan.

Kata kunci: Catharanthus roseus, embrio matang, hipokotil, node batang, organogenesis, somatik embrio

1 INTRODUCTION

Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don, synonym *Vinca rosea* L., *Lochnera rosea* Reich.) is a tropical perennial plant, native to Madagascar, belonging to Apocynaceae family (Gupta, 1977; Kohlmüzer, 1968). Presently, it is planted as ornamentals and widely grown in many tropical and subtropical regions. The main reason of a great interest in *C. roseus* is its ability of synthesize a wide range of terpenoid indole alkaloids (TIAs) which are valued due to their wide spectrum of pharmaceutical effects. Among the 100 different indole alkaloids isolated from different parts of the plant, vincristine (VCR) and vinblastine (VLB) are more important and better known. The anticarcinogenic indoles, VLB and VCR are used in leukemia and lymphomas and the alkaloids are present in leaves of the plant (Barbara Łata, 2007).

It was reported (Verpoorte *et al.,* 1993) that the cost of VCR was about US $ 3,500/g and that of VLB about US $ 6,800/g. These alkaloids are found to produce in trace quantities or are not at all in undifferentiated systems such as the callus and suspension cell culture (Pietrosiuk *et al.*, 2007). VLB and VCR occur in very low concentrations of 1 g t^{-1} and 20 mg t^{-1} of plant material, respectively (Tyler, 1988) but they are still considered as the most interesting chemotherapeutic compounds currently accessible for clinical use (Pezzuto, 1997).

The therapeutic and economic importance of the dimeric alkaloids vinblastine and vincristine has stimulated the research on cell biotechnology of *C. roseus* in countries in the temperate regions notably in the Netherlands, Poland, Japan and South Korea where the species may be only grown as annual crop in the open field, using transplants raised in a greenhouse or in a plastic tunnel (Buchwald, 2007). In contrast, in Malaysia with the tropical climatic condition where the species can be grown all year round has great

potential for the species to grow in large scale for their highly valued pharmaceutical properties.

The low yields of the anticarcinogenic alkaloids in the plant, combined with their high market price have encouraged intense research for alternative methods for the production of these alkaloids (Moreno *et al.*, 1995). The use of plant cell cultures for the production of secondary metabolites is an approach which has many attractions. Its application to alkaloid production by *C. roseus* is not, however, straightforward (Parr *et al.*, 1988). Another approach to enhance the production of these alkaloids in *C. roseus* plant is by developing transgenic *C. roseus* plant with increased expression of genes involved in the biosynthesis of these alkaloids (Zárate & Yeoman, 2001).

As an integral part of transgenic technology, *in vitro* plant regeneration is now being pursed more actively (Sim & Cardosa, 2005). Plant regeneration via somatic embryogenesis and callus culture is reported as a potential way for the production of transgenic plants (Lorence & Verpoorte, 2003). In the meantime, methods of organogenesis are also important for production of transformed plants, as the introduction of DNA into plant cells implies the posterior differentiation of these cells into a whole plant (Marguerite, 2003). True-to-type propagules are the expected product of clonal propagation whether from organogenic or somatic embryogenic regeneration (Adriana & Wagner, 2003). Genetic stability is thus a prerequisite for the application of transformation systems.

The formation of somatic embryos is a unique mode of *in vitro* plant regeneration and offers many advantages, including the potential for unlimited production of clones with elite traits, to be used as medicinal raw material for secondary product (Junaid *et al.*, 2006). However, in many other plant species somatic embryogenesis is found to be

genotype specific (Sim & Cardosa, 2005). Different varieties could response differently in culture. Therefore there is a need to develop an *in vitro* plant regeneration system for our local varieties of *C. roseus* for possible development of a transgenic *C. rose*us plant with increased yield of vincristine or vinblastine in future.

The objective of this project is to develop an *in vitro* plant regeneration system in local cultivars of *C. roseus* which then can be applied in the widely used *Agrobacterium*-mediated plant transformation system. This project also aimed to study the differences in the response to the induction of somatic embryogenesis and shoot organogenesis in different cultivars.

2 LITERATURE REVIEW

2.1 The plant

Catharanthus roseus (L.) G. Don is a perennial evergreen herbaceous or semi-shrub plant belonging to the Apocynaceae. The common name of this species is Madagascar periwinkle or the Common periwinkle. It is also called Vinca, Rose periwinkle or Rosy periwinkle. In Jamaica it is better known as Ram-goat rose or Old maid in Grenada, or Caca poule in Dominica, or as periwinkle in most West Indian places. This species was classified by the Swedish botanist Linnaeus as *Vinca rosea* L. in 1735 and in 1828 by Reichenbach as *Lochnera rosea* Reichbach. In 1838, G. Don established the genus as *Catharanthus* and its taxonomy was defined in 1956 (Kohlmüzer, 1968).

The species was originally native to Madagascar and India. It has been widely cultivated for hundreds of years and now is found growing wild in most warm regions of the world. *C. roseus* produces a large number of flowers, nearly year-round, under tropical conditions. Plants are fully self-fertile and self-pollinated with a high heritability but automatic self-pollination does not normally occur in periwinkle (Sreevalli *et al.*, 2000). Pollination in the field is carried out mainly by butterflies. In natural sites of occurrence propagation by seeds is common. Again the tropical and subtropical zones have been found to be the most suitable for cultivation of *C. roseus*. The seeds are sown directly into the soil (Barbara Lata *et al.*, 2007).

Within the species of *C. roseus* different forms or varieties exist with respect to growth, development, morphogenesis and alkaloid related characters (Mishra *et al.*, 2001). The colour of flowers could be from white to purple (Plates 1 and 2) and the height of plants varies from 1 to 2.5 m. Leaves are ovale-eliptical or lanceolate with an acute or dub-acute apex and they are placed alternately in opposite position on the shoots. Their upper

surface is lustrous, with clearly visible nervure. Pentatherous flowers develop single, in the angles of leaves, most abundantly in the apical parts of the shoots. The basal part of petals is blended into a tube. Sepals are lanceolate, sharply ended and several times shorter than petals and also blended at the base. The flower has five stamens and one pistil, composed of two carpels. The stigma is covered with gluey hairs (Buchwald *et al.*, 2007). Fruits of periwinkle have an elongated form, usually slightly curved pouch, up to 4.5 cm in length. Botanically the fruit is known as 'silique'. They are green, but at ripening get dark blots at the surface. Fully ripened fruits drop easily, pouring out the seeds. Depending on growing conditions, from several to several tens of fruits are developed on a single plant. One pouch contains about 20 seeds, on the average. Seeds are black, with verrucose surface and concave on the middle of one side like an 'eye'. The root system of periwinkle is composed of a strong main root and numerous lateral roots, usually thin and curved. Roots are yellow.

According to Staszewski *et al.* (2007), seeds harvested at 'yellow silique' stage of maturity were in high germination rate where the yellow siliques were still steadily attached to the stem and were not shed down; nevertheless they were easy to pick by hand. Seeds inside siliques were black. The 'brown silique' stage was considered too late for harvesting, because most of the siliques cracked at this stage of maturity and considerable portion of seeds could be lost. The stage of 'green silique' was however, too early for harvesting of seeds. All seed characteristics were significantly unfavourable at that stage, resulting in a poor overall seed quality. Therefore, it is most appropriate to pick the 'yellow silique' for *in vitro* seed germination.

Bar =1.5cm

Plate 2.1 *Cathranthus roseus* cv. W-Y
White petal with yellow centre

Bar =1.5cm

Plate 2.2 *Cathranthus roseus* cv. P-Dp
Purple petal with dark purple centre

2.2 The use of the plant

The species has long been cultivated as an ornamental plant and for herbal medicine. As an ornamental plant it is gaining greater popularity as horticulturists have developed varieties with a wider range of flower colours as well as growth habit. Now there are annual, small, prostrate and trailing types. They are grown as borders and beds, as edging plants and ground covers. It is great in porch planters and window boxes.

As medicinal plant, *C. roseus* has long been used as folk remedy in India, China, Central and South America and Europe. Although not explicitly stated, the discovery of potent substances in *C. roseus* in 1957 (Benard, 1967) was probably a major reason for the changes in attitude towards research on plants used in traditional medicine. The discovery of oncolytic properties of the alkaloids in *C. roseus* has in 30 years resulted in hundreds of scientific reports and stimulated the search for other antitumour agents of plant origin (Leeuwenberg, 1987). It is of considerable interest to note that the first report on *C. roseus* was dated back to more than three hundred years. The first printed information on the species given in 1658 in De Flacourt's. 'The comparison to the Asclepias' is interesting that though it is obviously based more on medicinal properties of the plant than on

morphology, it nevertheless indicated a relationship that had later been confirmed by systematists.

Early pharmacological studies (Noble *et al.*, 1958) were done in Canada on the leaves of the white-flowered plants sent by Dr. C. D. Johnson from Black River in Jamaica. The plant extracts were tested for the reputed antidiabetic activity and the results were disappointing. Subsequently, the range of tests was expanded. It was then that the fortuitous discovery was made that carcinostatic activity sided in the plant extracts. *C. roseus* is the most important source of anti-cancer drugs, such as vincaleukoblastine (Duke, 1985) but West Indian people still believe that teas made from the plant can cure diabetes and hypertension.

As the most important plant source of anticancer drugs, *C. roseus* is consumed in U.S. at an estimated 1000 MT, with another 1000 for England, Italy, Netherlands and West Germany. The principal alkaloid is vincaleukoblastine (Vinblastine sulfate), sold as Velban. Vinleurisine, vinrosidine, and vincristine possess demonstrable oncolytic activity. Extracts of the plant show beneficial growth inhibition effects in certain human tumors. Vinblastine sulfate is used experimentally for treatments of neoplasm, and is recommended for generalized Hodgkin's disease and resistant chorricarcinoma. Vincristine sulfate (Oncovin) is used in treatment of leukemia in children. Using vincristine and vinblastine in combination with chemotherapy has resulted in 80% remission in Hodgkins disease, 99% remission in acute lymphatic leukemia, 80% remission in Wilm's tumor, 70 % remission in gestational choriocarcinoma and 50% remission in Burkitt's lymphoma (Junaid *et al.*, 2005).

2.3 Pathways of *in vitro* plant regeneration

2.3.1 Regeneration from existing meristems and adventitious meristems

Plant regeneration from existing meristems is a useful method of quick biomass production for pharmaceutical purposes. Plant regeneration can be done through proliferation of axillary shoots from pre-existing meristem. Elevated cytokinin levels are usually required for the induction of shoots. Their effect has been demonstrated in many cases but how these signals are translated into morphogenic responses have remained obscure so far (Charriere *et al.*, 1999).

Shoot multiplication either directly or through a callus phase can be obtained by inducing adventitious shoot production on mature plant organs such as leaves, petiole and internode. These require the initiation of *de novo* meristems, which may be controlled by the balance of auxin and cytokinin in the culture medium. Regeneration from adventitious meristems may significantly enhance the potential multiplication rate of plant. Thus the presence of morphologically competent cells that under appropriate chemical and physical conditions give rise to shoots in culture means that high multiplication rates may be achieved and that mechanization of the handling of cultures is a possibility. However, there may be problems of genetic instability in that the progeny arising from single cell may not be true-to-type. Polyploidization is the most common occurrence and is attributed to selection of preexistent cells (Fowler & Warren, 1992).

The first observations related to the formation of roots from the callus tissue of *C. roseus* were reported by Dhruva *et al.* (1977). Ramavat *et al.* (1978) described the formation of shoots of *C. roseus* from the callus. The multiple shoot cultures of *C. roseus* were directly induced, with high frequencies, from seedlings on the MS medium (Murashige & Skoog, 1962) supplemented with BA 1.0 mg/L (Hirata *et al.*, 1987). The

resulting cultures also consisted of unorganized tissue on the solid medium and multiple shoots having several small leaves.

Moreno *et al.* (1995) prepared a review article which summarized the progress made on cell and tissue cultures of *C. roseus*, covering the literature cited in the Chemical Abstracts from 1988 up to December 1993. The interest in *C.roseus* has remained high and increasing number of publications and patents concerning a large spectrum of applications of these cultures. However, no commercial production of alkaloids by cell cultures has been achieved. The technology for alkaloid production by large-scale cultivation of plant cells is available. Nevertheless, for an industrial large-scale production the alkaloid levels need to be improved. In order to reach the goal a better understanding of the regulation of secondary metabolism is needed.

Z´arate *et al.* (1999) reported that adventitious organogenesis of buds and shoot induction were achieved using differentiated tissue (nodal explants bearing two axillary buds) as a source of plant material. The reason of choosing nodal explants is due to the recalcitrant nature of *C. roseus* callus or suspension cultures for regeneration. The highest number of buds (9.0) was recorded on 1 mg/L BAP medium (½ MS 1.0) after 36 days. The number of buds induced increased steadily with time in the different media (namely ½ MS 0.5 & ½ MS 0.75); however, the trend did not show statistically significant differences except at day 36 for the highest BAP-containing medium. In addition, shoot morphology was similar for shoots derived from either medium.

Hernández-Domínguez *et al.* (2004) describes a protocol for the induction, maintenance, and characterization of a vindoline-producing *C. roseus* shoot culture. Shoots are formed from seedling explants within the first 10 days of culture. After three weeks, an average of eight shoots per explant had been formed on semisolid MS media,

supplemented with 3% sucrose and 1.0 mg/L of BA. The shoots had been maintained for up to 12 months, without noticing callus or root formation. In contrast, shoots induced by 3.0 and 5.0 mg/L BA tend to form callus and roots after long culture periods (usually more than 6 weeks). Shoots proliferated mainly from axial buds and took between four and eight days to unfold. The number of shoots formed per explant using 1.0 mg/L of BA is the same, either under continuous light or under a 16-h photoperiod. However, photoperiod shoots are smaller than those under continuous light. Therefore, shoots had been transferred to fresh semisolid MS media every four weeks and maintained on 1.0 mg/L of BA, under continuous light. Under these conditions, a single shoot can form up to 10 new shoots after three weeks.

Plant regeneration was also obtained in *C. roseus* hypocotyls. Genotype specificity and combinations of growth regulators were found to be important for plant regeneration (Choi *et al.*, 2004). Hypocotyl explants produced hairy roots when cultured on MS basal medium after infection by *Agrobacterium rhizogenes*. Explants gave rise to adventitious shoots at a frequency of up to 80% when cultured on MS medium supplemented with 7 mg/L of 6-benzyladenine and 1 mg/L of α-naphthaleneacetic acid. There was a significant difference in the frequency of adventitious shoot formation for each hairy-root line derived from a different cultivar. Plants derived from hairy roots exhibited prolific rooting and had shortened internodes. Approximately half of the plants had wrinkled leaves and an abundant root mass with extensive lateral branching, but otherwise appeared morphologically normal. Plants with hairy roots that were derived from the cultivar Cooler Apricot developed flowers with petals that were white in the proximal region, whereas the wild-type flower petals are red.

Pietrosiuk *et al.* (2007) presented an overview of studies using various plant tissues, organ culture and plant regeneration of *C. roseus*. The authors stated that plant

regeneration was accomplished by somatic organogenesis. Studies conducted in different highly specialized laboratories have demonstrated that in many cases the ability to produce secondary metabolites is associated with the process of organogenesis. The finding has contributed to considerable progress in the field of micropropagation.

Dhandapani *et al.* (2008) established an efficient regeneration system via somatic embryogenesis and organogenesis using five different types of explants of *C. roseus* cv. Little Bright Eye: mature embryo, cotyledon, etiolated hypocotyl, shoot tip, and stem node. Mature embryo showed a better response to TDZ than other explants, giving rise to the highest percentage of adventitious shoot (87.7%) and somatic embryo (48.7%) at 0.5 mg/L and 1.5 mg/L of TDZ, respectively. Shoot tip and stem node were highest in shoot organogenesis at 0.5 mg/L BA plus 1.0 mg/L NAA which gave 87% regenerated shoots with an average of eight and ten shoots per explant. Hypocotyl and cotyledon did not induce somatic embryogenesis and organogenesis in TDZ-containing medium but gave a maximum percentage of shoots in MS medium supplemented with 1.0 mg/L NAA and 0.5 mg/L BA.

2.3.2 Regeneration by somatic embryogenesis

Recovery of transgenic plants requires transgene(s) to be delivered to tissues capable of regenerating to produce fertile and phenotypically normal plants under tissue culture conditions. Regeneration via somatic embryogenesis is advantageous for genetic manipulation and propagation due to the single cell origin and bipolar growth habit of the embryos. Such cultures consist of rapidly proliferating tissues in which totipotent cells are located on the surface of small embryogenic units. All the plantlets produced from embryogenic cells have the same genetic makeup. Combined with genetic engineering,

micropropagation through somatic embryogenesis provides an efficient means of producing a large number of elite or transgenic plants.

Plant regeneration of *C. roseus* cv. Little Delicata was obtained from anther-derived callus via somatic embryogenesis (Kim *et al.*, 1994). Calli were obtained from anthers cultured on MS medium supplemented with 1.0 mg/L α-naphthalene acetic acid and 0.1 mg/L kinetin. After the second subculture on solid medium, embryogenic callus was identified and transferred to liquid medium to initiate suspension cultures. Upon plating onto the basal medium, yellowish compact colonies proliferated from the cells and more than 80% of them gave rise to somatic embryos. Subsequently, plantlets developed from the embryos. Both the plantlets and the source plants showed the normal somatic chromosome number of 2n=2x=16.

Kim *et al.* (2004) described the culture conditions for plant regeneration in immature zygotic embryo-derived embryogenic cell suspension cultures of *C. roseus* cv. Little Bright Eye. Immature zygotic embryos formed off-white, friable calli at a frequency of 20% on MS medium supplemented with 1.0 mg/L 2,4-D after eight weeks of culture. After a second subculture using MS basal medium at 4-week intervals, off-white friable calli formed a small quantity of yellowish, compact embryogenic calli. Upon transfer to MS basal medium, embryogenic calli gave rise to numerous somatic embryos. Cell suspension cultures were established with embryogenic calli using liquid MS medium supplemented with 1.0 mg/L 2,4-D. Embryogenic cell clumps from cell suspension cultures developed into plantlets at a frequency of 56.7% when plated onto MS basal medium.

Roles of some external factors in proliferation, maturation and germination of embryos in *C. roseus* have been discussed by Junaid *et al.* (2005). The process of

embryogeny, particularly the aspect of maturation, germination or plantlet conversion, is a complex mechanism of interdisciplinary nature involving embryology, physiology, biochemistry and other subjects. Although many of the facts have been addressed quite successfully in recent times, there are still questions that remain unanswered. Reduction in structural abnormalities will definitely increase the regenerability of somatic embryos. Besides, proper embryo selection and their transfer to optimized germination medium, selection of rooted plantlets and their transfer to soil for acclimatization are some of the important stages and/or cultural practices that need more attention for success and reproducibility of plantlet production. The origin of the embryo is said to be from a single cell, which is easily amenable to genetic modification.

Junaid *et al.* (2006) has developed an efficient somatic embryogenesis method in *C. roseus*. Friable embryogenic callus was induced from hypocotyl of *in vitro* germinated seeds on MS basal nutrient media supplemented with various auxins particularly 2,4-D (1.0 mg/L). However, only NAA (1.0 mg/L) produced somatic embryos in cultures. Embryo proliferation was even high on the same medium added with BAP. Cotyledonary somatic embryo germinated and converted into plantlets in BAP (0.5 mg/L) added medium following a treatment with GA_3 (1.0 mg/L) for maturation. Carbon sources and concentrations had a marked influence on maturation process. Plantlet conversion was better achieved when embryos were matured on 3% fructose or 3–6% maltose. Before transfer *ex vitro*, plantlets were cultivated on half strength MS medium containing 3% sucrose and 0.5 mg/L BAP for further development of new shoots. The authors indicated that somatic embryos were produced in numbers and converted plantlets can be used as raw material, genetic modification to embryo precursor cell may improve alkaloid yield further.

Both primary and secondary/adventive somatic embryogenesis and the role of plant growth regulators in two modes of somatic embryo formation have been further discussed by Junaid *et al.* (2007). Hypocotyl-derived embryogenic callus (HEC) was friable and fast-growing, while secondary callus derived from primary cotyledonary somatic embryos (PCSEC) was compact and slow-growing. HEC differentiated into somatic embryos which proliferated quickly on medium supplemented with NAA (1.0 mg/L) and BA (1.5 mg/L). Although differentiation and proliferation of somatic embryos were faster in primary HEC, maturation and germination efficiency were better in somatic embryos developed from PCSEC. At the biochemical level, two somatic embryogenesis systems were different.

Junaid *et al.* (2008) evaluated the roles of various factors in different stages of embryogenesis in *C. roseus*. There are a number of plant growth regulators (PGRs) that are used to induce somatic embryos (SEs) during culture and the right balance or the ratio of these PGRs is often the primary empirical basis for the optimization of *in vitro* SE development. Aside from PGRs, the most commonly used carbohydrate for plant tissue or cell culture is sucrose, but this is not always the best carbohydrate to achieve regeneration. In order to be beneficial, certain changes in carbohydrate sources are often necessary. There are several other factors (pH, sugar alcohols, activated charcoal, light, polyethylene glycol, amino acids, solidifying agents, etc.) which also influence embryogenesis.

3 MATERIALS AND METHODS

The main objective of this project was to develop an *in vitro* plant regeneration system in local cultivars of *C. roseus* which can be applied in the widely used *Agrobacterium*-mediated plant transformation system. This was done through two pathways of plant regeneration which are direct organogenesis and somatic embryogenesis. Mature embryo, leaf, petiole, stem node and hypocotyl were used as explant to identify which of these are more amenable to plant regeneration when provided with suitable nutrients and growth conditions. Furthermore, different cultivars were used to study the differences in the response to the induction of somatic embryogenesis and shoot organogenesis. Plant material was the mature seeds and seedlings derived from surface sterilized seeds and grown aseptically in the Plant Tissue Culture (PTC) laboratory in the Faculty of Resource Science and Technology (FRST), UNIMAS.

3.1 Sterilization and *in vitro* germination of seeds

Mature fruits or 'Yellow siliques' of *Catharanthus roseus* (L.) G. Don were collected from plants of specific cultivars grown naturally in the open. The seeds isolated from the siliques were washed in running water for 5 minutes and surface sterilized in 70% ethanol for 2 minutes followed by treatment with a freshly prepared solution of 7% (v/v) Clorox (5.25% sodium hypochlorite) with the addition of one drop of Tween 20 (approximately 0.01%) to the Clorox solution for 10 minutes. The seeds were rinsed five times with sterilized double-distilled water and blot-dried with sterile tissue paper. Sterilized seeds of about 20 to 25 in number were sown on each of the disposable Petri dishes (87 mm x 15 mm) containing 25 ml half-strength MS (Murashige & Skoog, 1962) mineral salts.