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AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION IN *DENDROBIUM*

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Thesis submitted in partial fulfillment of the requirement
for the degree of

Doctor of Philosophy in Horticulture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2005

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DECLARATION

I hereby declare that the thesis entitled "***Agrobacterium* mediated genetic transformation in *Dendrobium***" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled **"*Agrobacterium* mediated genetic transformation in *Dendrobium*"** is a record of research work done independently by Smt. R. Swarnapiria under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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ACKNOWLEDGEMENT

I owe a deep sense of gratitude and heartfelt salutation to the chairman **Dr. K. Rajmohan**, Associate Professor & Head, Department of Pomology and Floriculture, College of Agriculture, Vellayani who has been an inspiration in pursuing this study. The meticulous guidance, valuable suggestions and solid support at all stages during the course of study and in the preparation of this thesis are strongly acknowledged.

I am bound in gratitude to the member of the Advisory committee, **Dr.K.B.Soni,** Assistant Professor, for her valuable counselling, timely suggestions and help rendered by her. I express my profound thanks to **Dr.M.M. Viji**, Assistant Professor, Department of Crop physiology for her constant encouragement, kind counselling and help rendered by providing some chemicals required for the study.

I acknowledge **Dr. T. Sabeena George**, Associate Professor, Department of Pomology and Floriculture for her kind suggestions and help during the course of study and thesis preparation. I am grateful to **Dr. V.L. Sheela**, Associate Professor, Department of Pomology and Floriculture for her valuable suggestions, which improve the exposition of this thesis.

I have no words to express my feeling of gratefulness to **Tamilnadu Agricultural University, Coimbatore** for granting study leave and financial assistance during the course of study.

My heartfelt thanks and gratitude belongs to **Dr. Balasubramanian**, Professor & Head, Department of Biotechnology and **Dr. Sudhakar**, Professor, Department of Biotechnology, TNAU, Coimbtore for their kind help in providing the different strains of *Agrobacterium* and plasmids for my study.

I acknowledge **Dr. Adelheid Kuehnle**, Professor of Horticulture, University of Hawaii, Honolulu, for the literatures and valuable suggestions offered during this study.

I thank **Dr.M.G. Purushothama**, Scientist, Rajiv Gandhi Centre for Biotechnology, Trivandrum for his kind help and providing some important chemicals and helped in conducting some important experiments. I thank Dr. C.R. Ananthakumar, Professor, Department of plant Breeding, Agricultural College & Research Institute, Killikulam for his valuable suggestions and constant encouragement.

I thank ***Dr. Lekha Rani, Dr. Uma Maheswaran, Dr. Sivaprasad, Dr. Lalu Das, Dr. Roy Stephen, Dr. Manju and Dr. Krishna Prasad*** of the College of Agriculture for providing the materials required for different experiments whenever I approached them. I thank ***Dr. C.S. Jayachandran Nair, Dr. B.K. Jayachandran Nair, Dr. B.R. Reghunath, Dr. Abdul Vahab, Dr. Rajamani and Dr. Sulekha*** of the College of Agriculture, Vellayani for their kind help rendered during the course of study.

I thank my friends ***Thangaselvabai, Anita, Sima, Simi, Priyakumari, Pradeep, Satheesh, Suseela, Suresh, Rajiv and Sudhir,*** who had helped me in this study.

I express my irremediable gratitude to my parents **Mr. K. Ramakrishna Pillai** and **Mrs. N. Mathavi Ammal** for their moral support. I thank my husband **Er. M.S. Ragavan** for his co-operation and kind help. I express my thanks to my dear son **R. Shivaram** for his patience which helped me in the preparation of this thesis.

Finally I express my profound thanks to **Vee Jay Computer Centre**, Nagercoil for the Computer typing of this manuscript.

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LIST OF ABBREVIATIONS

AC	-	Activated charcoal
BA	-	Benzyladenine
CW	-	Coconut water
CD	-	Critical difference
CH	-	Caesin hydrolysate
½ MS	-	Half-strength Murashige and Skoog
MS	-	Murashige and Skoog (1962) medium
NAA	-	1-Naphthyl acetic acid
pH	-	Per hydrogen
PLBs	-	Protocorm like bodies
VW	-	Vacin and Went (1949) medium
Vir	-	Virulence genes
v/v	-	Volume by volume
npt	-	Neomycin phosphotransferase
hpt	-	Hygromycin phosphotransferase
gus	-	β – D – glucuronidase
kb	-	Kilobase pairs
bp	-	Basepairs
DNA	-	Deoxy ribonucleic acid
DNTPs	-	Deoxy nucleotide triphosphates

EDTA	-	Ethylene diaminotetra acetic acid disodium salt
M	-	Molar
μM	-	Micromolar
OD	-	Optical density
PCR	-	Polymerase chain reaction
TAE	-	Tris acetic acid EDTA
Tris-HCl	-	Tris (hydroxy methyl) aminomethane hydrochloride

INTRODUCTION

Orchid flowers are the most fascinating among all ornamentals. They are highly priced and they account for 8.0 per cent share of the global floricultural trade, worth 44 billion US dollars, with an annual growth rate of 10 to 20 per cent. Orchids exhibit remarkable diversity in form and growth habit *viz.*, terrestrial, epiphytic, lithophytic, saprophytic and even sub-aquatic. The attractive flower forms also make these perennials one of the strongest choice for breeding programmes. Bewildering colours, shapes and sizes of these flowers, persistence of bloom and their ability to withstand long distance transport made these flowers one of the top ten cut flowers in the international market. Potted blooming orchids are gradually becoming an important feature of orchid business worldwide. Besides the ornamental value, orchids are used for medicinal purposes and a large number of alkaloids have been isolated from them. The first one, *dendrobine* was isolated from *Dendrobium nobile*. Besides, orchids are valued as jewellery, decorating dresses and also for decorative weavings.

India is home to about 1300 orchid species, a large number of them are important floriculturally. A substantial number of Indian indigenous species figured prominently in international breeding programmes to develop superior orchid hybrids. India's varied climatic conditions offer it an unique advantage over the other orchid-growing countries of the world. All tropical, temperate and intermediate types of orchids can be grown easily in the country. Thus, India has a great potential of becoming a major exporter of orchids at global level. Its main strength lies in the rich gene pool, varied climatic zones, availability of trained scientific man power, good institutional infrastructure, lesser cost of production compared to that in the other major orchid-growing countries.

Among the different epiphytic orchids grown in India, *Dendrobium* has its prime position owing to its free flowering habit and attractive blooms which are in high demand in the domestic and international market. More than hundred species of *Dendrobium* are grown in India. *Dendrobium* hybrids are the most suitable and popular among the commercial orchids grown in Kerala. Some of the popular *Dendrobium* hybrids grown here are Sonia, Sabine, Ekapol, Emma White, Thailand White, Kasem White, Walter Omae, Mary Trowse and Candy Stripe. Sonia (*D. caesae x D. Tomie Drake*) is the most free flowering among the *Dendrobium* hybrids.

Breeding of *Dendrobiums* using traditional methods like hybridization is restricted due to the long growth cycle (three years from seed to flower), slow seed maturation process and difficulty in seed germination *ex vitro*. One effective alternate method is the molecular breeding or genetic transformation. Advances in the recombinant DNA technology provide new opportunities for manipulation of the genome. Genetic engineering is entering a period of very rapid application and offers a means of expanding the market for ornamentals in the near future (Rajmohan, 2003).

Genetic transformation can be attempted either by direct delivery of genes into plant cells by specific methods such as particle bombardment, electroporation etc., or by indirect method through the mediation of *Agrobacterium tumefaciens*. Genetic modification of *Dendrobiums* for disease and stress resistance, precocious flowering, improvement of flower colour and floral architecture is of commercial importance. So far, there are only a few reports on the genetic transformation of *Dendrobium*.

The first work on genetic transformation of orchids was taken up in 1992 by Kuehnle and Sugii (1992) and the earlier works were only on direct DNA transfer by particle bombardment. The poor suitability of *Agrobacterium* for infection of this monocot was assumed as a major limitation, in the late nineties. Further difficulties

encountered by workers are that the orchid cells had a low rate of proliferation, *in vitro*. Second, the orchid cells are recalcitrant to tissue culture manipulations and plant regeneration from de-differentiated cells had not been achieved for orchids (Yang *et al.*, 1999). Further, the orchids cells in tissue culture exude a large quantity of phenolics that become toxic to the cells when oxidized. Orchids are resistant to the commonly used aminoglycosides such as kanamycin and often require high dose of antibiotics to select the transgenic cells, and in most cases sensitivity response can be detected only after five weeks. All these hurdles led to limited scientific work in this aspect. But in this millennium several workers had undertaken *Agrobacterium* mediated transformation system in orchid to standardize the various requirements for transformation, as orchids are substantially different from other plants in their requirements for a transformation system. Hence, the present study was undertaken with the main objective of standardizing the various techniques for *Agrobacterium* mediated genetic transformation in *Dendrobium*, Sonia 17.

2. REVIEW OF LITERATURE

Methods of traditional breeding have been used in the production and improvement of the diverse array of commercially available orchids. There remains a huge reservoir of traits including flower colour, scent, size, floral architecture, precocious flowering nature, growth habit, stress tolerance and disease and pest resistance that may be amenable for further genetic enhancement of orchids. *Dendrobiums* are one of the leading cut and potted floricultural crops grown in the tropics. Breeding of *Dendrobium* using hybridization is restricted due to the long generation time (generally three years from seed to flower), slow seed maturation process, and lack of useable genetic variability. One effective alternate procedure for *Dendrobium* improvement is based on gene transfer technology either by direct delivery of genes into the plant cells or by indirect methods through the mediation of *Agrobacterium*.

The first report on plant genetic transformation using *Agrobacterium tumefaciens* was in tobacco (Bewan *et al.*, 1983). Since then, this gene delivery system has been widely used for the transfer of desirable genes in several crop plants like cotton, tomato, sunflower, beans etc. Success of *Agrobacterium* mediated transformation has been reported to be dependent on the variety, type of explants, delivery system, *Agrobacterium* strain, conditions of co-cultivation, selection method and mode of regeneration (Mathis and Hinchee, 1994). Earlier, *Agrobacterium* mediated transformation was considered difficult in monocotyledons. Recent advances in the understanding of the biology of the infection process and the availability of efficient gene promoters and selectable markers improved the progress of genetic transformation in monocotyledons (Smith and Hood, 1995).

The identification and availability of genes of horticultural interest are increasing, and molecular breeding therefore appears as an attractive method for varietal improvement in orchids. In addition, increasing consumer interest in florist products worldwide signals that tremendous economic benefits may be gained by the creation of new and improved orchid hybrids. As a result, applied projects on orchid molecular biology and genetic engineering were being pursued initially with *Phalaenopsis* and *Neofinetia* in Japan, *Dendrobium* at the University of Hawaii, Honolulu (Kuehnle and Sugii, 1992; Nan and Kuehnle, 1995), *Cymbidium* in Korea, *Phlaenopsis* at the Taiwan Sugar Research Institute, *Calanthe* at the US Department of Agriculture, Beltsville (Griesbach, 1994) and *Dendrobium* at the National University of Singapore in conjunction with Rockfeller University (Chia *et al.*, 1994). The application of genetic transformation technique in orchids is fairly young and there are only a few reports on this aspect.

2.1. *IN VITRO* PRODUCTION OF PROTOCORMS

For molecular breeding to be feasible, the tissue to be genetically engineered must give rise to plants. In *Dendrobium,* the protocorm derived from the seeds, excised shoot tip, lateral buds, and protocorm-like bodies (PLB) derived from tissue explants are the tissues suitable for reliable orchid plant production. Protocols for the establishment of cultures *in vitro* and direct formation of plants from these meristematic tissues are available for many orchid genera (Arditti and Ernst, 1993). Other tissues capable of plant regeneration like etiolated shoots are suitable for *Dendrobium* hybrids (Kuehnle, 1997).

Protocorms are derived from germinating seeds. Sterilisation prior to inoculation of orchid seeds is very important, as orchid seeds have to be cultured under completely aseptic conditions for the development of protocorms. Since mature orchid seeds have tough seed coats, chemical treatments for sterilization can be safely employed (Jordan, 1965).

2.1.1 Surface Sterilisation of Pods

Green pod culture was proved to be the best, as the seeds directly transferred to the medium without exposure to the outside germinated well and produced strong seedlings within eight to ten weeks (Rao and Avadhani, 1964). Mature seeds of *Vanda* Miss. Joaquim pretreated with 5.0 per cent Chlorox for ten minutes and rinsed with sterile water prior to inoculation produced seedlings in ten to twelve weeks whereas mature seeds without pretreatment were lost due to contamination. Mitra (1971) used chlorine water to sterilise capsules and seeds. Pods were dipped in absolute alcohol (12 seconds) and chlorine water (45 minutes), whereas seeds folded in filter paper were dipped in chlorine water for ten minutes and rinsed with three changes of sterile water.

Rosa and Laneri (1977) used 70 per cent ethanol for sterilising pods. Pyati and Murthy (1995) achieved pod sterilization in *Dendrobium ovatum* by dipping in alcohol followed by flaming. Pod sterilization of *Vanda coerulea* was effected by pretreatment in 0.1 per cent mercuric chloride for five minutes, followed by alcohol dip and flaming.

2.1.2 Seed Germination and Development *In Vitro*

Knudson (1946) showed that the seeds of *Cattleya, Laelia* and *Epidendrum* germinated freely on sugar and mineral containing agar medium under aseptic conditions without fungal association. Arditti (1979) reported in four orchid genera including *Dendrobium* that only a few apical cells of protocorms divided to form a promeristem which gave rise to shoot apex and structures homologous to cotyledons. Mathews and Rao (1985) reported that the differentiated protocorms had to be subcultured within a period ranging from 70 to 80 days for proper *in vitro* growth. Overcrowding without transfer resulted in stunted growth. According to Yam and Weatherhead (1988) the seeds had germinated only when protocorms either green or

white, were observed in cultures. Rubulo *et al.* (1989) defined germination as the presence of protocorms with one leaf primordium one month after culture. Pathak *et al.* (1992) in *Goodyera biflora* reported that the protocorms, on emergence from the testa were white and hairy. The first signs of chlorophyll development were apparent in leaf initials. Singh (1992) reported that depending upon their genotype, the seeds develop chlorophyll within 10-20 days on the nutrient medium.

Krishnan *et al.* (1993) observed the visible protocorm formation from the embryos by the second and third week of culture in *Spathoglottis plicata* and the first leaf primordium was intitiated between the fifth and sixth week of culture.

Nagashima (1993) studied the seeds of 47 orchid species and reported that the germination rate ranged from 0.8 to 100 per cent and the number of days from sowing to germination ranged from 3 to 305 depending on the stage of embryogenesis. Singh (1993) found that inoculation of seeds into a nutrient medium under *in vitro* conditions not only improves the per cent of germination, but also reduces the time for differentiation of orchid seeds, both biochemically and morphologically.

Hazarika and Sarma (1995) reported that immature seeds of *Dendrobium transparens* showed the signs of swelling, in 16-18 days after inoculation. Ninety per cent germination was observed after 25 days of inoculation. Lekharani (2002) reported that in *Dendrobium* the seed germination per cent ranged from 8.00 to 70.73.

2.1.3 Effect of Culture Media on Seed Gemination

Many media have been used for the axenic germination of terrestrial and epiphytic orchids. However, none of these media is universal.

The commonly used nutrient media for orchid seed culture are those proposed by Knudson (1946) (KC), Vacin and Went (1949) (VW) Murashige and Skoog

(1962) (MS), Raghavan and Torrey (1964), Nitsch (1969), Mitra *et al.,* (1976) and Rosa and Laneri (1977) (RL).

Seed germination and morphogenesis studies in *Epidendrum radicans* and *Dendrobium* Jaquelyn Thomas clearly indicated the superiority of MS medium over KC and VW media (Sangama, 1986). Reddy *et al.* (1992) observed that the South Indian orchids exhibited a significant interaction between the media and the species. *Dendrobium crepidatum* yielded better results in MS and RL media than in KC medium. Zhang *et al.* (1993) reported that since MS medium contained high ionic concentration of nutrient salts half-strength MS could adequately support rapid protocorm production in orchids. Hazarika and Sarma (1995) conducted *in vitro* germination studies in *Dendrobium transparens* (Lindl) and reported that best growth of seedlings was obtained in supplemented MS medium.

Bhaskar (1996) found that supplemented quarter strength MS could produce seedlings with maximum number of shoots, leaves and roots in *Phalaenopsis* after a 12 week culture period. The basal medium MS half strength was found to be the best for early germination and rapid *in vitro* development as compared to MS quarter strength and MS, KC and VW full strengths (Lekharani, 2002). Xiang *et al.* (2003) reported that the best medium for *in vitro* regeneration of *Cymbidium sinensis* was MS medium supplemented with 4.0 mg BA + 1.0 mg NAA / l.

Devi *et al.* (1990) pointed out that the prefered medium for *Dendrobium* seed germination varied with the species. *D. farmeri* and *D. Primulinum* gave 50-60 per cent higher germination on VW medium.

Kumaria and Tandon (1991) were of opinion that high ionic concentration of nutrient salts and vitamins in the medium was inevitable for the germination of *Dendrobium fimbriatum* var *Oculatum* seeds. On inoculating four-month old seeds,

the highest germination (91%) was obtained on Nitsch medium, followed by MS (85%).

2.1.4 Effect of Organic Additives on Protocorm Establishment

The most frequently used complex organic additive in the production of orchid protocorms is coconut water (CW), the liquid endosperm of coconut. It induces cell division in otherwise non-dividing cells and promotes morphogenesis and mass multiplication of protocorms in orchids (Intuwong and Sagawa, 1973).

Morel (1974) had enumerated the beneficial effects of coconut water in bringing about rapid protocorm multiplication in orchids. Mc Intyre *et al.* (1974) found that addition of coconut water (15%) to KC medium led to increased growth of both epiphytic and terrestrial orchids. Vigorous root growth was observed in epiphytes. Coconut water (10%) when added to KC medium along with micronutrients, gave satisfactory germination in five orchid genera (Rosa and Laneri, 1977). Sahid (1980) reported that growth rate of *Dendrobium* hybrids could be improved by adding potato and pea extracts to KC medium.

Soediono (1988) found that supplemented VW medium (CW 15% + NAA 10 ppm) led to rapid protocorm proliferation followed by enhanced seedling growth in *Dendrobium* Jaquelyn Thomas. According to Rubulo *et al.* (1989) supplementing KC medium with 10 per cent coconut water gave the best germination in *Bletia Urbana.* Addition of 15 per cent CW enhanced germination and accelerated seedling growth in *Dendrobium farmeri* and *D.Primulinum* (Devi *et al.*, 1990). Immature seeds of *Rhyncostylis retusa* and *Vanda Coerulea* gave 20 per cent enhanced germination when VW media was supplemented with CW, banana pulp, pineapple juice and vitamin stock of Nitsch medium (Nath *et al.*, 1991).

Sharon *et al.* (1992) used the basal medium supplemented with 15 per cent CW for raising protocorms of *Dendrobium* Snowfire from immature seeds. Enhanced growth in different orchids has been reported to occur in the presence of coconut water (CW), banana pulp, peptone, apple juice and peptone, fish extract and peptone, pineapple and tomato fruit (Arditti and Ernst, 1993). For *Cattleya, Encyclia* and *Oncidium* 25 per cent CW was the best additive (Villolobes and Munoz, 1994).

Bhasker (1996) had pointed out the beneficial effects of peptone and CW on *in vitro* seedling growth in *Phalaenopsis.* Peptone 1000 mg l^{-1} along with BA 20 mg l^{-1} and NAA 1 mg l^{-1} maximised shoot leaf and root production after 12 weeks of culture. Foliar growth was enhanced by the addition of CW. Lekharani (2002) reported that coconut water 200 mgl^{-1} was the best for early protocorm differentiation and rapid seedling growth. According to Mathews and Rao (1980) yeast extract was successfully used for seed germination and protocorm proliferation in many orchid species.

2.1.5 Effect of Charcoal on Protocorm Establishment

Ernst (1974), Rosa and Laneri (1977) recorded that seedlings grew well on culture media to which activated charcoal was added. Fridborg *et al.* (1978) attributed the beneficial effects of activated charcoal to its adsorption of inhibitory phenolic and carboxylic compounds produced by the tissues in culture. They further observed that charcoal has the tendency to absorb hormones and vitamins and thereby inhibit growth. Hence it should be used with caution in culture media. The initial formulations of charcoal-containing medium for seed germination of Hong Kong orchids gained wide acceptance (Yam and weatherhead, 1988).

Pierik *et al.* (1988) found that in *Paphiopedilum ciliolare* when activated charcoal 2 gl^{-1} was added to the medium after protocorm formation, induced significant increase of shoot and root development. But it was inhibitory during seed

germination. According to Hinnen *et al.* (1989) activated charcoal strongly enhanced the growth and development of *Phalaenopsis* seedlings. Yam *et al.* (1990) observed that activated charcoal exerted a beneficial effect on culture media by adsorption and removal of phytotoxic metabolites. They further pointed out that it can also be detrimental due to the removal of additives such as auxins or cytokinins.

2.1.6 Effect of Carbon Source on Seed Germination

Orchids must have an external supply of carbohydrates to continue their growth and differentiation. Orchid seeds and young seedlings have the ability to utilize various carbohydrates. However, different species have their own preferences (Arditti, 1967). Glucose, fructose or oligosaccharides containing these sugars alone could adequately satisfy the energy requirements of *Phalaenopsis* protocorms (Ernst *et al.,* 1971). In *Dactylorrhiza purpurella*, the results with dextrose and sucrose were essentially similar (Harvais, 1972).

Of the sugars tested on the growth of *Cymbidium* protocorms, sucrose was better than maltose, glucose and fructose. The optimum concentration of sucrose ranged from 3.0 to 4.0 per cent (Fonnesbech, 1972). Harrison and Arditti (1978) found that sucrose induced germination and enhanced chlorophyll development in certain species that failed to germinate on sugar-free medium. Sucrose could be replaced by glucose.

In hybrid *Vanda,* Mathews and Rao (1985) and in *Cypripedium reginae,* Bellard (1987) tested different carbon sources and found that 2.0 per cent sucrose was the best source. Absence of sucrose stopped the growth of protocorms and 10.0 per cent sucrose caused tissue necrosis. Pierik *et al.* (1988) concluded that in *Paphiopedilum ciliolare* an extraordinary low sugar concentration was optimal for germination, higher concentration being inhibitory.

High sucrose concentration (4.0) reduced germination in *Bletia Urbana,* but no significant difference could be observed in the response between 2.0 and 3.0 per cent sucrose (Rubulo *et al.,* 1989). Sharma and Tandon (1990) reported that among the various carbon sources tested, sucrose, fructose and glucose at 2.0 to 3.0 per cent gave the best germination and seedling growth in *Cymbidium elegans* and *Coelogyne* sp. In sugar-free medium, the germination and growth were negligible.

Lekharani (2002) observed that sucrose 30 gl^{-1} showed significantly early development of first leaf and root primordia. Xiang *et al.* (2003) reported that 30g sugar/l was the optimum dose for the *in vitro* regeneration of *Cymbidium sinensis.*

2.1.7 Effect of pH Of Media on Seed Germination

Knudson (1951) noted the inability of *Cattleya* seeds to germinate if the initial pH of medium is below 4.5. *Dendrobium nobile* germinated better within a pH range of 4.0 to 5.0. (Ito, 1955) where as many other orchid species responded favourably to media with pH between 5.0 and 6.0 (Scott and Arditti, 1959; Kotamori and Murashige, 1965). Maintaining the pH at 5.2 to 5.5 was favourable for successful germination in *Cymbidium mastersii* (Prasad and Mitra, 1975).

Rosa and Laneri (1977) observed that pH of 5.2 for *Cattleya,* and *Phalaenopsis* and 6.0 for *Cymbidium* and *Paphiopedilum* were satisfactory for germination. Reyburn (1978) recorded in *Cymbidium* that germination in the dark was optimal at pH 5.5 – 6.0 and a pH of 7.0 was strongly inhibitory. Orchid seeds germinated well within a pH range of 4.8 to 5.2 with germination commencing at pH 3.6 and tapering off at 7.6 (Arditti, 1979).

Maximum germination and optimal growth of protocorms at pH 5.0 was reported in *Dendrobium chrysanthum* and *Sarcanthus pallidus* (Raghuwanshi *et al.,* 1986). Optimal germination of *Paphiopedilum ciliolare* occured at a pH of 6.0

(Pierik *et al.,* 1988). Ichihashi (1990) obtained good germination of *Blettia striata* seeds when the pH was adjusted to 5.1 ± 0.1. George (1997) found that optimal growth of protocorms in *Dendrobium osterholt* resulted when the pH was adjusted to 5.8.

2.2 ESTABLISHMENT OF PROTOCORM LIKE BODIES

Protocorm like bodies (PLBs) are obtained from the culture of shoot apices *in vitro.* Sagawa and Shoji (1967) opined that shoot tip cultures necessiated the sacrifice of the entire new growth or a whole plant for a procedure which at best might be successful with 66.7 per cent of the explants. As with *Cymbidium* and other sympodials shoot tips remained the most commonly used explant (Goh, 1970; Teo *et al.*, 1973).

Stewart and Button (1975) reported that plantlets and callus which subsequently gave rise to plantlets could be differentiated from a single *Paphiopedilum* stem apex if bacterial-free cultures could be obtained. Shoot tip explants of *Dendrobium fimbriatum* produced a compact callus after two weeks of inoculation. Following transfer to plant growth regulator free medium, the callus further proliferated with side by side regeneration of PLBs (Jonojit and Nirmalya, 2003).

The totipotent callus of *Cypripedium formosanum* an endangered slipper orchid was induced from the seed derived protocorm segments. The callus proliferated well and on an average 13 PLBs were obtained from a piece of 4 mm callus (Lee and Lee, 2003).

2.2.1 Effect of Culture Media on the Development of PLBs

The most commonly employed media for shoot tip culture are Knudson's C (Knudson, 1946), MS (Murashige and Skoog, 1962), and VW (Vacin and Went 1949).

Irawati *et al.* (1977) reported that the best growth and survival rates were obtained in *Dendrobium* when cultured in Knudson's C medium. Shoot tip explants of *Dendrobium fimbriatum* produced PLBs when cultured on modified nutrient solution of Knudson's C to generate the PLBs (Jonojit and Nirmalya, 2003).

Based on studies with *Aranda, Cattleya, Dendrobium* and *Ascocentrum* in three different media, Fu (1978) found that the best medium for proliferation was MS salts. Lee and Lee (2003) used quarter strength MS medium for producing the PLBs from the totipotent callus of *Cypripedium formosanum* Inflorescence tips of *Mokara* Cv Chark Kuan cultured on Vacin and Went medium produced PLB's (Abdulkarim and Hairani, 1992).

2.2.2 Effect of Plant Growth Substances on the Development of PLBs

When excised apices of *Rhyncostylis gigantea* were cultured on a composite agar medium supplemented with NAA and coconut milk, plantlets could be produced in three and half months (Vajrabhaya and Vajrabhaya, 1970). A higher concentration of NAA and BA induced maximum proliferation of shoots in *Cattleya* (Kusumoto, 1979). Addition of low concentration of NAA (below 0.1 mg / 1) promoted shoot formation (George and Sherington, 1984). Callusing was recorded in the presence of 0.5 mgl^{-1} NAA and 1 mgl^{-1} BAP. But the callus proliferated with side by side regeneration of PLB's when it was transfered to plant growth regulator free medium (Jonojit and Nirmalya, 2003).

Application of BA to the axillary buds increased the shoot proliferation in *Dendrobium antennathum* (Kukulczanka and Wojciechowska, 1983). Shimasaki and Uemoto (1987) found that application of BA to axillary bud explants of *Calanthe* promoted shoot growth.

Ichihashi (1992) reported rapid proliferation of lateral buds on young flower stalks of four hybrids of *Phalaenopsis* cultivar in the absence of growth regulator.

The totipotent callus of *Cypripedium formosanum* was induced from the seed derived protocorm segment on a quarter strength MS medium containing 4.52 μM 2, 4-D and 4.54 μM thidiazuron. The callus proliferated well and was maintained by subculturing on the same medium. On an average 13 PLBs were obtained from a piece of four mm callus after being transferred to the same medium with 4.44 μM BA after eight weeks of culture.

2.2.3 Effect of Carbon Source on the Development of PLBs

According to Hew *et al.* (1988) and Hew and Math (1989) when apical meristems of *Dendrobium* were cultured in a VW medium, fructose was more readily utilised than other sugars. Honjo *et al.* (1988) observed that the increase in fresh weight of PLB's of *Cymbidium* was markedly affected by sucrose concentration. The beneficial effect of CO_2 enrichment was observed only in the case of low sucrose concentration.

Sucrose [5.0% (w/v)] concentration was found to be the most effective in shoot induction (Paek and Yeung, 1991). Sucrose 3.0 per cent was found to be the best for shoot-growth from shoot meristems of *Dendrobium* Joannie Ostenhault (Madhuri and Vasundhara, 1990).

2.2.4 Effect of Organic Additives on the Development of PLBs

Coconut water has been proved to promote the growth and differentiation of excised tissues and organs of several crops. The beneficial effect of coconut water has been attributed to the presence of cytokinin like substances.

The optimum concentration of coconut water in the medium was 10 - 15 per cent. It is added before autoclaving (Morel, 1965, Intuwong and Sagawa, 1973). Coconut water 15.0 per cent differentiated more number of plantlets within a short period in *Dendrobium* (Soediono, 1983). In *Dendrobium fimbriatum* modified nutrient solution of Knudson's C supplemented with 10% V/V coconut water, 0.5 mg l^{-1} niacin and 0.5 mgl^{-1} pyridoxine HCl resulted in the production of compact callus which further proliferated into PLBs (Jonojit and Nirmalya, 2003).

Kusumoto (1979) reported that yeast extract retarded organogenesis and accelerated the production of protocorms in *Cymbidium.* Addition of 5.0 per cent pineapple juice to VW medium enhanced germination and accelerated leaf and root growth (Devi *et al.*, 1990).

Agarwal *et al.* (1992) found that in *Vanilla walkeri* MS medium supplemented with 0.5 mg l^{-1} kinetin, 1.0 mg l^{-1} BA and 1000.0 mg l^{-1} caesin hydrolysate supported rapid proliferation of multiple shoots from stem node segments.

2.2.5 Effect of Gelling Agent on the Development of PLBs

Kusumoto (1980) reported that in KC basal solution containing 15 gl^{-1} agar, *Cymbidium* protocorms proliferated best when 10 to 25 per cent coconut milk was added. PLBs from inflorescence tips of *Mokara* were cultured on solid Vacin and Went medium supplemented with 0.5 to 7.0 per cent agar. Increasing the agar concentration above 1.4 per cent resulted in reduction in the number of plantlets and

leaves (Abdulkarim and Hairani,1992). Xiang *et al.* (2003) reported that the best concentration of agar for *in vitro* regeneration of *Cymbidium* was 8 g l^{-1}.

2.2.6 Effect of pH of the Medium on the Development of PLBs

The pH in the medium greatly influences plantlet growth (Knudson, 1951). *Vanda* explants were grown best on White's medium with pH 5.5 (Sagawa and Sehgal, 1967). *Epidendrum* leaf tips were best grown at pH 5.5 on modified MS medium (Churchill *et al.,* 1970). Mosich *et al.* (1974) recommended a pH of 5.5 for *Dendrobium*. The same author reported a higher pH of 5.8 when modified MS medium was used for *Dendrobium* culture.

Epidendrum root tips were reported to grow on modified Ojima and Fujiwara medium with a pH of 5.0 (Churchill *et al.*, 1972). *Cattleya* shoot tips when cultured on a solid medium turned brown and died eventually. Tests conducted on polyphenol oxidase activity showed that the leaves tuned brown due to this activity. The activity was greatest at pH 6.5 and was inhibited at lower pH (Ichihashi and Kako, 1977).

The genes bracketed by the border sequence in a T-DNA, eventhough they are of prokaryotic origin, contain eukaryotic promoters and regulatory sequences. As a result, these genes do not express inside the bacterial cell. It is precisely because of this, the genes encoded by the T-DNA can be replaced without interfering with the transfer of the T-DNA to the plant cell. *Agrobacterium* is able to transfer 10-20 kb DNA to the plant cell. The T-DNA is organised in two distinct regions called TL (left T-DNA) and TR (right T-DNA). The removal of oncogenes from T-DNA of the Ti plasmid and replacement with the desired gene permits the use of this bacterium for the genetic transformation of plant tissue (Bernard and Jack, 2001).

2.3 MARKER AND REPORTER GENES

Selection of transformed cells is a key factor in developing a successful genetic transformation system (Chia *et al.,* 1994). Single dominant genes encoding suitable resistance to a selective agent is used as a marker. The reporter genes are used to analyse the function of promoters and other gene regulatory sequences. These genes do not disrupt the plant regeneration, but allow the selection of transformed cells.

Neomycin phosphotransferase II *(npt II)* gene from transposon Tn 5, detoxify neomycin, kanamycin and G 418 by phosphorylation. It is widely used in dicotyledon system, including tobacco, potato and tomato (An *et al.*, 1986), legumes such as white clover (White and Greenwood, 1987) pea (Puontikaertas *et al.*, 1987) and woody species such as *Pseudostuga menziesii* (Ellis *et al.*, 1989).

The *'bar'* gene codes for phosphinothricin acetyltransferase (PAT) which inactivates PTT, an irreversible inhibitor of glutamine synthase. This gene has been inserted and expressed in rape (De Block *et al.,* 1989), rice (Dekeyser *et al.*, 1989) and alfalfa (Krieg *et al.*, 1990).

Hygromycin phosphotransferase *(hpt II)* governs resistance to hygromycin. This gene isolated from *E.coli* has been placed under various promoters and has been successfully used in rice (Dekeyser *et al.*, 1989; Shimamoto *et al.*, 1989). Dekeyser *et al.* (1989) evaluated the efficiency of various selectable markers. They reported that while phosphinothricin and bleomycin were effective at lower concentrations, G 418 and hygromycin were required at higher concentrations for selection of transformed rice cells. Nehra *et al.* (1990) had reported the use of *hpt II* gene as a marker in strawberry.

An alternative to antibiotic selection is the use of the firefly luciferase gene, *luc* (Chia *et al.*, 1994) as a marker. This was used in *Dendrobium* orchid. The product of this gene produces light upon reaction with luciferin, which can be detected with a camera photomultiplier.

The green fluorescent protein (GFP) is efficiently expressed in plant cells and it is used as a selectable marker by Mercuri *et al.* (2001) in *Lisianthus* and in *Dendrobium*, Sonia 17 by Tee *et al.* (2002).

2.4 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

There is a substantial interest in the genetic improvement of orchids. Orchids form the largest family of flowering plants with more than 800 genera and over 25,000 species that are commercially grown globally (Arditti, 1992). Genetic modification of orchids for disease and stress resistance, precocious flowering and the improvement of flower colour and morphology is thus of major commercial importance. The advent of transgenic technology allows for accelerated modification and improvement of orchids.

In orchids the first report on genetic transformation was made by Kuehnle and Sugii (1992). They obtaint transgenic *Dendrobium* plants from protocorm like bodies (PLBs) using particle bombardment. Professor Chia was the first to genetically engineer an orchid with firefly luciferase gene (Chia *et al.,* 1994).

2.4.1. Explant for Genetic Transformation

For molecular breeding to be feasible the tissue to be genetically engineered must give rise to plants. In turn, the gene transfer method and the gene expression vectors to be used must be compatible with the plant genotype and the tissue to be treated. Integral to the process should be a means of identifying and selecting for

organ genesis from engineered tissue. Together, these factors determine the effectiveness of a particular plant genetic engineering system.

Kuehnle and Sugii (1992) recovered stable transformants of *Dendrobium* Jaquelyn Thomas from the protocorms bombarded by microparticles coated with the plasmid, PGA482GG/cp PRV4). Outgrowths resembling protocorms from cultured leaf segments of *phalaenopsis* were used as explants to generate stable transformants by Anzai *et al.* (1995). Nan and Kuehnle (1995) reported the electroinjection of 50 – 100 protocorms of *Dendrobium* UH 44 inbred, aged two to three months, with PBI 121 DNA, to generate the transformants. Chen and Kuehnle (1996) obtained transformed plants in *Authurium* by cocultivation of etiolated internodes with *A. tumefaciens*.

PLB of *Dendrobium* White Angel were bombarded with tungsten microparticles coated with PUC 19-LUC or pMON530-LUC (Chia *et al.*, 1994) and the bioluminescent orchid was obtained. A cocultivation method was developed for transforming *Phalaenopsis* varieties *in vitro* with *Agrobacterium tumefaciens* using PLBs as explants (Hsieh *et al.*, 1997).

One transgenic plant of *Dendrobium* Jaquelyn Thomas 'Uniwai Blush' (UH 44) was recovered from an etiolated shoot explant bombarded with PBI 121 coated tungsten particles (Nan and Kuehnle, 1995). According to Vergauwe *et al.* (1998) transformation of *Artemisia annua* was accomplished by co-cultivation of sterile leaf, stem and root explants from 12 to 18 week-old plants, cotyledons and hypocotyls from eight day old seedlings with *A. tumefaciens.*

Protocorms of orchid (*Dendrobium* hybrid) were transformed by microprojectile bumbardment (Yu *et al.*, 1999). Yang *et al.* (1999) used the PLBs to transform the *Cymbidium orchid* using particle bombardment. Protocorms and protocorm like bodies of three genera *Brassia, cattleya* and *Doritaenopsis* were

genetically transformed *via* micro projectile bombardment (Knapp *et al.*, 2000). Transgenic orchid (*Dendrobium* Madame) plants were obtained by the co-cultivation of thin-section explants from PLBs with *A. tumefaciens* (Yu *et al.*, 2001).

Leaf tissues were used as target explants for establishing a stable transformation system for the ornamental plant *Datura meteloides* (Curtis *et al.*, 1999). Leaf derived embryogenic calli were inoculated with *A. tumefaciens* for producing transgenic plants of *Agapanthus praecox* ssp. *orientalis* (Suzuki *et al.*, 2001). Leaf discs were co-cultured with *A. tumefaciens* to generate the transformants in chrysanthemum (Shinoyama *et al.*, 2002), in cape daisy (Morbel *et al.*, 2002) and in rhododendron (Dunemann *et al.*, 2002). Gerbera leaves with two to three mm lamina lengths were transformed by *A. tumefaciens* LBA 4404 (Korbin *et al.*, 2002).

Callus initiated from the *in vitro* grown cormel slices of *Gladiolus* Cv Jenny Lee and the suspension cells were bombarded to generate transgenic plants (Kamo and Blowers, 1999). Cormels of 1.0 to 1.5 cm diameter cut into 2-3 mm thick slices, and *in vitro* derived bisected shoot tips were used as target explants for *Agrobacterium* mediated genetic transformation of gladiolus (Babu and Chawla, 2000). Belarmino and Mii (2000) obtained genetically transformed plants of *Phalaenopsis* orchid after co-cultivation of cell clumps with *A. tumefaciens*. Li *et al.* (2002) used undifferentiated callus, and primary embryogenic callus as explants for *Agrobacterium* mediated genetic transformation in rose. Three different morphological callus types identified as type A, B, C and tips of *in vitro* inflorescenses were used as target tissues, for genetic transformation of *Dendrobium* Sonia 17 (Tee *et al.*, 2002).

Genetically transformed plantlets of *Phalaenopsis* were obtained after co-cultivation of PLBs with *Agrobacterium tumefaciens* strain LBA 4404 containing the

vector PTOK 233 that harbours genes for β-glucuronidase (GUS) and hygromycin resistance (Chai *et al.*, 2002).

Transgenic plants of *Cymbidium niveomarginatum* were regenerated after co-cultivation of their rhizome sections with *A. tumefaciens* (Chan *et al.*, 2003). In *Arabis* hypocotyl explants were cocultivated with *Agrobacterium tumefaciens* strain GV 3101 (Taskin *et al.*, 2003). *Dendrobium phalaenopsis* and *D. nobile* were genetically transformed by particle bombardment using calluses and PLBs as target explants (Men *et al.*, 2003).

Liau *et al.* (2003) used PLBs from protocorms as target explant for the *Agrobacterium* mediated genetic transformation of *Oncidium*. For Phalaenopsis Cv Taisuco Crane the PLBs were used as explants for the two types of genetic transformation (Chan *et al.*, 2003). The effective genetic transformation for five carnation cultivars was established and optimized using the leaf explants, by Lin *et al.* (2003).

2.4.2. *Agrobacterium* Infectivity

Agrobacterium infectivity is a result of the interaction between the plant cell and the bacterial cell. The infectivity is improved by the use of right strain of the bacterium, varying host genotype, manipulating explant physiology, inoculation and co-cultivation conditions (Godwin *et al.*, 1992). *Agrobacterium* mediated transformation has been reported to be dependent on the variety, type of explants, delivery system, *Agrobacterium* strain, conditions of co-cultivation, selection method, and mode of regeneration (Mathis and Hinchee, 1994).

Earlier, *Agrobacterium* mediated transformation was considered difficult in monocotyledons. Recent advances in the understanding of the biology of the infection process, and the availability of gene promoters and selectable markers

improved the progress of genetic transformation in monocotyledons (Smith and Hood, 1995). *Agrobacterium* mediated gene transfer is usually generalised to produce simpler integration patterns, less rearrangements within inserts and reduced problems with cosuppression and instability over generations, compared to methods based on direct gene transfer (Komari and Kudo, 1999).

In *Agrobacterium* mediated genetic transformation in barley most of the transgenic lines that expressed both marker and reporter genes showed simple integration patterns and Mendelian inheritance of the transgenes in T_1 progeny (Trifonova *et al.*, 2001). *Agrobacterium* based DNA transfer system offers many unique advantages such as the simplificity of *Agrobacterium* gene transfer. It allows a precise transfer and integration of DNA sequences with defined ends. It ensures a linked transfer of genes of interest along with the transformation marker. This method results in a higher frequency of stable transformation with many single copy insertions (Veluthambi *et al.*, 2003).

2.4.3. Strain Specificity

Agrobacterium tumefaciens mediated transformation had been successful with a broad range of dicotyledonous plants and few monocotyledons. There are differences in the susceptibility between species and even between cultivars and genotypes of the species. So the best method is to try transformation with different strains harbouring a good selectable marker, till we get the genotype / strain combination. Several *A. tumefaciens* strains varying in chromosomal background, *vir* helper plasmid and binary vector plasmid should be tested for their competence to transform. Chen and Kuehnle (1996) obtained transformed plants is *Authurium* by the co-cultivation of etiolated internodes with *A. tumefaciens* strain LBA 4404.

Hsieh *et al.* (1997) in *Phalaenopsis* developed a method for transformation *in vitro* with *Agrobacterium* strain EHA 105. In *Artemisia annua A. tumefaciens* strain

EHA 101, C 58C1 Rif Rb (Vergauwe *et al.*, 1998) and in *Datura* the supervirulent *A. tumefaciens* strain 1065 were used to get transformants (Curtis *et al.*, 1999).

Belarmino and Mii (2000) used the strain LBA 4404 (PTOK233) and EHA 101 (pIG121Hm) for cocultivation of cell clumps to generate transformants. In *Chrysanthemum* cultivars (Seiun, Shohounotikara, Tenju and Houkou) *A. tumefaciens* strains EHA 101 and LBA 4404 with C 58C1 promoter was used to study the several factors including the chrysanthemum cultivars and *A.tumefaciens* strains which affect the stable transformation. They reported that EHA 101 was the best (Kudo *et al.*, 2002). In Cyclamen precultured etiolated hypocotyls of Cv Sierra Rose were infected with *Agrobacterium tumefaciens* strains LBA 4404 and EHA 105 to produce successful transformants (Boase *et al.*, 2002).

A. tumefaciens strain LBA 4404 was used to get successful transformants in gladiolus (Babu and Chawla, 2000) *Dendrobium* (Yu *et al.*, 2001), *Agapanthus* sp. (Suzuki *et al.*, 2001), African violet (Kushikawa *et al.,* 2001), chrysanthemum (Shinoyama *et al.,* 2002) and gerbera (Korbin *et al.,* 2002). In an ornamental plant *Osteospermum ecklonis* genetic transformation was performed using *A.tumefaciens* strain AGL 1 harbouring the expression vector p^{GREEN} under the control of the constitutive promoter 35S (Giovannini *et al.*, 2002).

(Kim *et al.*, 2002) reported the first successful *Agrobacterium tumefaciens* mediated transformation in *Alstroemeria* Cv VV024 by the infection of friable embryogenic callus lines and leaves with *A.tumefaciens* strain LBA 4404 (PTOK 233). The same strain was successfully used in *Phalaenopsis* (Chai *et al.*, 2002). In rose *A. tumefaciens* strain GV 3101 (Li *et al.*, 2002) and in *Coleus blumei* the wild type bacterial strain B 6 S3 gave maximum efficiency. The strains C 58 C 1, GV 3101, 8196 and A 281 were also effective (Bauer *et al.*, 2002).

Kishimoto *et al.* (2002) reported the transformation with young leaf discs of *Begonia* on infection by *A. tumefaciens* strain LBA 4404 and AGLO LBA 4404 was used to get the transformants in *Cymbidium niveomarginatum* (Chen *et al.*, 2002). In *Arabis gunnisoniana* the hypocotyl explants were co-cultivated with *A. tumefaciens* strain GV 3101 (Taskin *et al.*, 2003). *A. tumefaciens* strain LBA 4404 (pBIN19) was used to generate the transgenic dwarf and early flowering lilies (Mercuri *et al.*, 2003). LBA 4404 (pBIN19) generated transgenic African violets with antifungal properties (Ram and Mohandas, 2003).

2.4.4. Gene Transfer Methods

The gene transfer method and the gene expression vectors to be used must be compatible with the plant genotype and the tissue to be treated. Several gene transfer methods can be used. The methods tested with orchids include microparticle bombardment (Kuehnle and Sugii, 1992), seed imbibition (Chia *et al.*, 1994), pollen tube mediated DNA delivery and electroinjection (Nan and Kuehnle, 1995 a). Yu *et al.* (1999) transformed *Dendrobiums* and Yang *et al.* (1999) transformed *Cymbidiums* using particle bombardment.

Chen and Kuehnle (1996) obtained transformed plants in *Anthurium*, a monocot by co-cultivation of etiolated internodes with *A. tumefaciens.* In *Phalaenopsis* Hsieh *et al.* (1997) and in *Artemisia Vergauwe et al.* (1998) used *A. tumefaciens* for transferring the gene.

A stable transformation system was established in the ornamental plant *Datura meteloides* using *A. tumefaciens* (Curtis *et al.*, 1999). Belarmino and Mii (2000) in *Phalaenopsis* and Babu and Chawla (2000) in *galdiolus* sp Yu *et al.* (2001) in *Dendrobium* reported the *Agrobacterium* mediated genetic transformation. Transgenic *Agapanthus* (Suzuki *et al.*, 2001) and transgenic chrysanthemums (Shinoyama *et al.*, 2002) were by *A. tumefaciens* mediated genetic transformation.

A. tumefaciens mediated genetic transformation was reported in gerbera (Korbin *et al.,* 2002), rose (Li *et al.,* 2002); sedum (Yoon *et al.,* 2002), *Rhododendron* sp (Dunemann *et al.,* 2002) and *Phalaenopsis* (Chai *et al.,* 2002). In *Arabis* sp Taskin *et al.* (2003); carnation, Lin *et al.* (2003); *Oncidium* (Liau *et al.,* 2003) established an efficient *A. tumefaciens* mediated genetic transformation system.

Gladiolus Cv Jenny Lee was bombarded to generate transgenic plants (Kamo and Blowers, 1999). Knapp *et al.* (2000) transformed *Brassia, Cattleya* and *Doritaenopsis* by micro projectile bombardment. *Dendrobium phalaenopsis* and *D. nobile* (Men *et al.,* 2003) and *Dendrobium,* Sonia 17 (Tee *et al.* 2002) were genetically transformed by particle bombardment. Chan *et al.* (2003) reported transformation by both the methods viz., particle bombardment and sonication assisted *Agrobacterium* mediated transformation in *Phalaenopsis.*

2.4.5. Bacterial Density

Concentration of bacterial cells in the induction medium is another important factor to be considered for efficient transformation. Very low density of bacterial population could lead to ineffective transformation, whereas very high density may lead to necrosis and death of the explant. Some species are very sensitive to bacterial infection and hence very low density of bacterial population is used.

Curtis *et al.* (1999) reported in *Datura meteloides* that the treatments involving either a 1:20 (vol:vol) or 1:10 (vol:vol) bacterial dilution for inoculating explants regenerated ($P<0.05$) more GUS positive shoots than 1:5 (vol:vol) dilution. Belarmino and Mii (2000) followed a two step co-cultivation method. The first step involved the incubation of 1.0g suspension cells obtained from the friable calli of *Phalaenopsis* in 20ml solution consisting of 1:10 (vol / vol) *A. tumefaciens* suspension. After 10h of 30 rpm agitation at 28^0C all suspension cells were collected on a nylon mesh (20µM pore size), washed with sucrose free NDM medium and

blotted dry with sterile filter paper. In the second step the cells were spread on a piece of sterile filter paper placed on 20ml of co-cultivation medium consisting of NDM-20s supplemented with 500 µM acetosyringone and solidified with 0.8gl^{-1} agar in 90 x 20mm petriplates and co-cultivated for three days in the dark.

Agrobacterium suspension for co-cultivation was prepared by picking a single colony from a YEP plate and inoculated in 5ml of liquid YEP medium containing acetosyringone and antibiotics as per the binary vectors. After two days of growth the bacterial suspension were spun down (5000 rpm, 10min) and resuspended in MS medium supplemented with 100 µM acetosyringone. The OD of the culture was measured at A 600 nm and adjusted to 0.1 by dilution or concentration (Babu and Chawla, 2000).

Mishiba *et al.* (2000) used the bacterial suspension diluted to one-tenth concentration with the liquid callus induction medium. Lavender calli were put on a 40µM nylon mesh in a funnel and *Agrobacterium* suspension was poured on the calli, immediately blotted on filter papers and plated on agar-solidified callus induction medium for co-cultivation. Suzuki *et al.* (2001), attempted transformation from the cultured bacterial cells obtained from the bacterial suspension by centrifugation and resuspended in liquid MS medium and the final OD value was adjusted to 0.2. Embryogenic calli of *Agapanthus* were immersed into this bacterial suspension for one minute, and blotted on sterile filter paper and then co-cultivated to generate the transformants.

Seo *et al.* (2003) reported that in C*hrysanthemum* cv Puma, transformants were obtained when leaf discs were immersed *in A. tumefaciens* inoculated at 1:50 dilution ratio for five minutes. Chan *et al.* (2003) soaked the PLBs of *Phalaenopsis* in *Agrobacterium* inoculum for one hour and then co-cultivated the PLBs, to generate transgenic plants.

2.4.6. Genes Transformed by Various Methods.

A variety of gene expression vectors with selectable marker or reporter genes for identification of transformed shoots have been used in orchids. Genes of interest, including marker or reporter genes, are vectored on short units of DNA called plasmids. The plasmids have various gene promoters and other sequences which affect the ability of the plant cell to transcribe the gene and to translate it into a protein product. This, in turn, affects detection of transformation of the plant cell. Thus use of a plasmid with inappropriate promoters, marker or form (circular or linear) may mask the effectiveness of a specific gene transfer method in delivering DNA plasmids into cells (Kuehnle, 1997).

Stable transformants of *Dendrobium* Jaquelyn Thomas were recovered from protocorms bombarded by microparticles coated with the plasmid, pGA 482GG/ cp PRV4, which contains Nos-*npt II* encoded by *'neo'* gene and papaya ringspot virus coat protein genes (Kuehnle and Sugii, 1992).

Chia *et al.* (1994) transformed *Dendrobium* with the firefly luciferase gene, *luc*. The product of this gene produces light upon reaction with luciferin, which can be detected with a camera photo multiplier.

Phalaenopsis was bombarded by a pneumatic gene gun using gold microparticles coated with the plasmid pMSP 38 containing the *bar* gene, which codes for resistance to the herbicide bialaphos, driven by CaMV35S promoter. (Anzai *et al.,* 1995). Kamo and Blowers (1999) in gladiolus and Knapp *et al.* (2000) in three orchid genera, *Brassia, Cattleya* and *Doritaenopsis* transformed the *bar* gene from *Streptomyces hygroscopicus* calli of *Lilium formolongi* were bombarded with the plasmid pACT 1-F which harbour the *Uid A* (GUS) gene driven by the rice actin1 promoter and pDM 302 harbouring the *bar* gene and selected for bialaphos resistance (Irifune *et al.,* 2003).

One transgenic plant of *Dendrobium* Jaquelyn Thomas 'Uniwai Blush' (UH 44) was recovered from an etiolated shoot explant bombarded with PBI 121 (*npt II* and *gus A*) coated tungsten particles (Nan and Kuehnle, 1995). Hsieh *et al.* (1997) in *Phalaenopsis* varieties transferred the bacterial neomycin phospho transferase II *(npt II)* gene and B-glucuronidase *(GUS)* gene, as a selection marker and reporter gene, respectively.

Chen and Kuehnle (1996) obtained transgenic *Anthurium* plants with good disease tolerance by using *A. tumefaciens* containing ${}_{p}$ca 2 A++, ${}_{p}$ca213 and ${}_{p}$ca274 vectors.. These vectors carry genes for antibacterial peptides, cecropia attacin, phage p22 and phage T 4 lysozyme gene. Protocorms of *Dendrobium* hybrid were transformed by microprojectile bombardment. Gold particles coated with plasmid DNA containing GUS and hygromycin phospho transferase (*hpt*) marker genes were used (Yu *et al.,* 1999). Belarmino and Mii (2000) genetically transformed *Phalaenopsis* by the co-cultivation of *A. tumefaciens* LBA 4404 (pTOK233) and EHA 101 (pIG121Hm) that harboured GUS and hygromycin resistance (*hpt*) genes.

Yang *et al.* (1999) introduced a plasmid DNA ($p^{KH\ 2001}$) carrying the *GUS* INT and *npt II* genes into the meristematic cells of PLBs by particle acceleration. Babu and Chawla (2000) co-cultivated the cormel slices and *in vitro* derived bisected shoot tips with *Agrobacterium* strain LBA 4404 harbouring the binary vectors PBI121 and pTOK 233 which contained *gus* reporter gene with rice actin and 35S promoters, respectively.

Cymbidium niveomarginatum were regenerated after co-cultivation of their rhizome sections with *A. tumefaciens* strains LBA 4404 with PBI 121 which has *gus* and *npt II* (Chen *et al.,* 2002). The ${}_{p}$CAMBIA 2301 plasmid containing the *Uid A* gene coding for *gus* gene and *npt II,* was immobilized into *A. tumefaciens* strain GV 3101 and used in rose (Li *et al.*, 2002). In *Cyclamen, A. tumefaciens* strains LBA 4404 and EHA 105 containing the binary vectors p MOG 410 or PART 27-10

was used. Both has *NOS-npt II* gene and 35s gus A intron (Boase *et al.*, 2002). In *Arabis gunnisoniana* the hypocotyl explants were co-cultivated with *A. tumefaciens* strain GV 3101 harbouring PBI 40 which contained *npt II*, as a selectable marker (Taskin *et al.*, 2003). The plasmid PIG121Hm, harboured *npt II, hpt* and *gus A* gene (Kudo *et al.*, 2002). This was transformed to chrysanthemum (Kudo *et al.*, 2002); begonia (Kishimoto *et al.*, 2002) saintpaulia (Kushikawa *et al.*, 2001).

In a cut flower plant O*steospermum ecklonis* Mercuri *et al.* (2001) introduced Green Fluorescent Protein (GFP) and is efficiently expressed in plant cells. Five different DNA plasmids carrying a synthetic (*gfp*) gene driven by different promoters CaMV35S, HBT and Ubil were tested for the efficiency of transformation. In *Dendrobium* Sonia 17, 35 ss gfp TYG–nos (P35S) with the CaMV35S promoter showed the highest GFP transient expression rate (Tee *et al.*, 2002)

In cape daisy, Morbel *et al.*, (2002) obtained virus resistant plants by transferring the genes for 6k2 and NIa (Nuclear Inclusion protein a). After the successful transformation with *GUS* gene as a reporter, non-chimeric plants could be obtained. Tomato spotted wilt virus (TSWV) was identified as a casual agent of an important disease in ornamentals. The TSWV nucleo protein gene was introduced into gerbera genome. An expression vector (containing the CaMV35S promoter, the selectable *npt II* gene and N-gene of the virus normally infecting gerbera) was constructed in $_P$BIN 19 plasmid and placed in *A. tumefaciens*, LBA 4404. gerbera explants (shoots, bases of shoot clumps, and 2-3mm length pieces of leaf lamina) were co cultivated with LBA 4404 to generate transgenic gerberas (Korbin *et al.*, 2002).

Shinoyama *et al.* (2002) transformed chrysanthemum Cv Shuho-no-Chikara using a disarmed strain of *A.tumefaciens*, LBA 4404, carrying a binary vector PIAbT$_1$, that harboured a *cry 1 Ab*gene encoding an insecticidal crystal protein fragment of *Bacillus thuringiensis.* A significantly higher feeding inhibition or

growth inhibition of insect was observed in transformed plants compared to those on the non-transformed control plants. A modified delta endotoxin gene modified *cry 1 Ab* of *Bacillus thuringiensis* was introduced into *Chrysanthemum.* Cv Shuho-no-Chikara. *A. tumefaciens* strain EHA 101 carrying a binary vector $_{P}$IG121 mcbt that harboured the *mcbt* gene encoding an insectidal crystal protein (ICP) fragment of *Bacillus thuringiensis* var Kustaki HD 1 was used. Transgenic plants showed significantly higher feeding inhibition compared to the untransformed (Shinoyama *et al.*, 2003).

Different combinations of antifungal defence genes were introduced into the garden rose cultivars, Heckenzauber and Pariser Charme, by Dohm *et al.* (2002). Eight true transgenics plants were analysed for the expression of their transgenes and resistance to black spot caused by *Diplocarpon rosae.* The secretion of the ribosome inhibiting protein into the extracellular space, however, reduced the susceptibility against black spot to 60 per cent on an average.

Kim *et al.* (2002) reported the first successful transformation in *Alstroemeria* Cv VV 024. They used *A. tumefaciens*, LBA 4404 with PTOK 233. The marker and reporter genes transformed were *hpt* and *gus,* respectively. Genetically transformed plantlets of *Phalaenopsis* were regenerated after co-cultivation of PLBs with *A. tumefaciens* strain LBA 4404 containing the vector PTOK – 233 that harbours genes *gus* and *hpt* (Chai *et al.*, 2002).

In *Phalaenopsis* Cv Taisuco Crane (Chan *et al.,* 2003); carnation (Lin *et al.*, 2003); *Oncidium* (Liau *et al.,* 2003) and *Dendrobium* (Men *et al.,* 2003), an efficient *A. tumefaciens* mediated genetic transformation system using *npt II* and *gus* gene as selectable marker and reporter genes, respectively was established. Condylife *et al.* (2003) optimised the *A. tumefaciens* mediated transformation of rose from embryogenic callus using the gus (Uid A) gene.

Petunia was transformed with *boers,* a mutated allele of BOERS, an ethylene receptor sensor gene of *Brassica oleracea.* Hygromycin resistant regenerants were tentatively confirmed as transformants. Transgenic flowers retained turgidity and pigmentation for longer time than the untransformed controls (Shaw *et al.,* 2002).

Rosati *et al.* (2003) modified the flower colour in *Forsythia* by introducing anthocyanin synthesis in petals through sequential *Agrobacterium* mediated transformation with dihydroflavonol 4 reductase from *Antirrhinum majus* (Am DFR) and anthnocyanidin synthase from *Matthiola incana* (MiANS) genes. The double transformants (Am DFR + MiANS) displayed a novel bronze orange petal colour caused by the *de novo* accumulation of cyanidin derived anthocyanins over the carotenoid yellow background of wild type (Wt) and intense pigmentation of vegetative organs.

African violet *(Saintpaulia ionantha)* leaf explants were inoculated with the strain LBA 4404 of *A. tumefaciens* harbouring the binary vector p^{BINAR} carrying glucanase-chitinase genes and *npt II* as selectable marker (Ram and Mohandas, 2003).

In *Lilium longiflorum* Cv Snow Queen, the embryogenic calli derived from flower styles and pedicels were inoculated and co-cultivated for seven days with a cell suspension of *A. tumefaciens* LBA 4404 harbouring the binary vector pBIN 19 containing the *npt II* gene driven by *NOS* promoter. The transformed plants were dwarf and early flowering (Mercuri *et al.,* 2003).

2.4.7. Co-cultivation

The explant chosen, in its most receptive stage, is exposed to the *Agrobacterium* culture in the induction media at an optimum bacterial density. Both the composition of the induction media and the time of induction play a key role in

the efficiency of transformation. For induction, regeneration medium of the explant which can support bacterial growth like MS medium is used. It can also be the bacterial culture medium. The P^H of the medium usually ranges from 5.5 to 5.7. For inoculation the explants are mostly immersed in the induction medium for a specific period of time, which depends on bacterial population, type of vector and type of explant used.

Hsieh *et al.* (1997) developed a co-cultivation method for transforming *Phalaenopsis* varieties *in vitro* with *A. tumefaciens* strain EHA 105. They reported that PLBs at the ten-day proliferating stage were the optimal materials for infection. Scanning electron microscopy (SEM) revealed that *Agrobacterium* attached very well to the surface cells of PLBs.

Murashige and Skoog salts and vitamins were used for the preparation of induction medium by Nagaraju *et al.* (1998) during their experiments with *Gerbera hybrida.* They immersed the explants in induction medium for five minutes and got successful transformants. In *Agapanthus* the embryogenic calli were immersed in the bacterial suspension for one minute and blotted on sterile filter papers. They were co-cultivated with *Agrobacterium* at 25^0C in the dark, for seven days. Several hygromycin resistant cell clusters were obtained (Suzuki *et al.,* 2001). In *Cymbidium niveomarginatum* higher efficiency of transformation was observed with 1.5 hours from the time of *Agrobacterium* infection and also within six days of co-culturing with *A. tumefaciens.* The *GUS* expression was almost 100 per cent. The GUS expression fell off sharply as the infection time and duration of co-culture increases. (Chen *et al.*, 2002). In *Coleus blumei* Bauer *et al.* (2002) reported that the incubation of excised leaf explants immediately in bacterial suspension for five minutes and then co-cultivation with *Nicotiana tabacum* crowngall callus for two days was the efficient treatment, which increased the transformation efficiency.

Curtis *et al.* (1999) reported that in *Datura meteloides* a co-cultivation period of two to three days, using a 1:20 or 1:10 v/v dilution of an overnight bacterial culture and in lavender (Mishiba *et al.,*2000) a co-cultivation period of two days using 1:10 dilution, resulted in transformed plants. The efficiency of transformation was markedly increased by co-cultivation of cell clumps with *A. tumefaciens* for ten hours together with 200 μM acetosyringone and by inclusion of 500 μM acetosyringone in the co-cultivation medium (Belarmino and Mii., 2000).

Yu *et al.* (2001) reported *Agrobacterium* mediated genetic transformation of *Dendrobium* with the class 1 knox gene DOH 1. The transformation was performed through two consecutive stages of co-cultivation, with the first stage occurring on antibiotic free medium for three days and the subsequent stage on medium containing 50 mgl^{-1} carbenicillin for 3-4 weeks. In *Saintpaulia ionantha,* Kushikawa *et al.,* (2001) generated transformants after 48 hours of co-cultivation with *A. tumefaciens* strain LBA 4404 (PTOK 233) harbouring the intron – gus A reporter gene and *hpt, npt II* as selective markers.

In *Chrysanthemum,* Kudo *et al.* (2002) reported that four days of co-cultivation at 24^0C was the optimum when using EHA101 and PIG121Hm plasmid. Co-cultivation of *Agrobacterium* for three days generated transformants from the leaf explants of *Sedum erythrostichum* (Yoon *et al.*, 2002). The embryogenic calli of *Lilium longiflorum* cv Snow Queen derived from flower styles and pedicels were inoculated and co-cultivated for seven days with a cell suspension of *A tumefaciens*, LBA 4404, harbouring the binary vector pBIN19 containing the *npt II* gene for kanamycin resistance, driven by *NOS* promoter. The transformed plants showed altered ornamental traits such as dwarfness and early flowering which are highly desirable (Mercuri *et al.*, 2003)

In *Oncidium* Liau *et al.* (2003) performed transformation through two stages of co-cultivation, one on antibiotic free medium for three days and a subsequent stage on medium containing $100mg^{-1}$ timentin for one month.

In *Phalaenopsis,* the PLBs were soaked in bacterial inoculum for one hour and then transferred to medium with 5 per cent glucose and 100μM acetosyringone and co-cultivated for three days. For sonication assisted *Agrobacterium* mediated transformation, the PLBs were sonicated for upto five minutes at 30 second intervals and then co-cultured with *Agrobacterium.* Maximum GUS expression was noticed when the PLBs were sonicated for 150 seconds and co-cultivated for three days at 26^{0}C (Chan *et al.*, 2003).

2.4.8. ADDITION OF ACETOSYRINGONE

Agrobacterium tumefaciens respond to certain phenolic compounds such as acetosyringone and hydroxyacetosyringone which are excreted by wounded plants. These small molecules act to induce the activity of virulence *(vir)* genes that are encoded on the plasmid. The *'vir'* genes are located on 35Kb region of the plasmid that lies outside the T-DNA region. When *A. tumefaciens* get attached to a plant cell, and the *'vir'* genes are induced, then 'T-DNA' (which contains the gene of interest) is transferred to plant cell.

Acetosyringone showed no effect and in some cases inhibitory effect on regeneration as in carrot (Pawlicki *et al.*, 1992). Torres *et al.* (1993) found no effect of acetosyringone or syringaldehyde during their transformation works in lettuce. Rashid *et al.* (1996) found that addition of acetosyringone in co-cultivation media did not help in attaining the objective of transformation and no *GUS* expression was obtained. On the other hand supplementing co-cultivation medium with tobacco feeder cells alone could give transient *GUS* expression. It may be presumed that

tobacco suspension culture contains some substances, other than acetosyringone which may facilitate T-DNA tranfer.

Babu and Chawla (2000) spun down the bacterial suspension after two days of growth, and resuspended in MSK medium (MS medium supplemented with 18.6mM Kinetin) supplemented with 100μM acetosyringone. Gladiolus explants were transferred to this medium and were incubated on a platform shaker at 100 rpm for 2 hours at 25^0C. Then the explants were blot dried on sterile filter paper and then co-cultivated for three days. Addition of acetosyringone generated transgenic gladiolus

The transformation frequency of lavender was improved by the presence of acetosyringone (100 μM) in co-cultivation medium. The effectiveness of acetosyringone might be assumed due to the v*ir* G gene harboured by pTOK233 was activated by the presence of acetosyringone (Mishiba *et al.*, 2000). Belarmino and Mii (2000) reported that the efficiency of transformation in *Phalaenopsis* was markedly increased by ten hour co-cultivation of cell clumps with *A. tumefaciens* that had been induced with 200 μM acetosyringone, and by inclusion of 50 μM acetosyringone in the co-cultivation medium.

Suzuki *et al.* (2001) obtained best results when embryogenic calli of *Agapanthus* were co-cultivated with LBA 4404/pTOK 233 for seven days in the presence of 20mgl^{-1} acetosyringone. Chan *et al.* (2003) soaked the PLBs in bacterial inoculum for one hour and then transfered them to a medium with 5.0 per cent glucose and 100 μM acetosyringone for co-cultivation for three days to obtain transformants in *Phalaenopsis.*

2.4.9. ELIMINATION OF BACTERIA AFTER CO-CULTIVATION

Complete elimination of bacteria from the explant after co-cultivation is very essential; otherwise it will interfere with the growth and organogenesis of the explant. Overgrowth of bacteria causes death of the explant and disrupts the experiment. Elimination of bacteria from the explant is done by the use of antibiotics. The antibiotic chosen should be such that it efficiently kills the bacteria; at the same time it does not affect the growth and organogenesis of the explants. The most commonly used antibiotics for this purpose are carbenicillin and cefotaxime. However, their effect on the explant has to be studied before choosing any one of them as they are also reported to have detrimental effect on some species.

Vergauwe *et al.* (1996) working on transformation of *Artemisia annua* L. found that cefotaxime at 50 mg l^{-1} was effective as a decontaminating antibiotic; but it caused retardation in callus formation. Then they tried vancomycin at 750 mgl^{-1} which was not toxic to the tissue. But it could not control the bacteria effectively. Belarmino and Mii (2000) used 300 mgl^{-1} cefotaxime to eliminate the bacteria after the co-cultivation of the *Phalaenopsis* cell clumps with *A. tumefaciens.*

After three days of co-cultivation with *A. tumefaciens,* the leaf explants of S*edum* were transferred to MS medium supplemented with $300mgl^{-1}$ cefotaxime to eliminate the bacteria (Yoon *et al.*, 2002). In *Datura* after two days of co-cultivation period, the leaf explants were transferred to the shoot regeneration medium supplemented with 200 mgl^{-1} kanamycin sulphate and 200 mgl^{-1} cefotaxime. Cefotaxime was used as the bacteriostatic agent (Curtis *et al.,* 1999). In *Agapanthus* 500 mg l^{-1} cefotaxime was used to eliminate *A.tumefaciens* after the co-cultivation of embryogenic calli (Suzuki *et al.,* 2001). Babu and Chawla (2000) used 100 mg l^{-1} cefotaxime after co-cultivation and incubated the explants for three days to arrest *Agrobacterium* growth.

In lavender the co-cultivated calli and leaf explants were transferred to the bacterial elimination medium, which was callus induction medium containing 500 mgl^{-1} cefotaxime. After seven days culture, the calli and leaf explants were transferred to the selection medium, which comprises callus induction medium containing 50 mgl^{-1} hygromycin and 200 mgl^{-1} cefotaxime (Mishiba *et al.*, 2000). In African violet, cefotaxime 800 mgl^{-1} was used to eliminate the *A. tumefaciens* strain LBA 4404 after co-cultivation of leaf explants (Ram and Mohandas, 2003).

2.4.10. Selection of Transformed Cells

Screening of untransformed cells or selection of transformed cells is an important aspect of transformation work (Chia *et al.*, 1994) several factors affect the choice of chemicals used for selection. The selection agent must be toxic to plant cells, though not so toxic that the products from the dying, non-tranformant cells kill adjacent transformed cells. Thus the most effective toxins are those which either inhibit growth of untransformed cells or slowly kill the untransformed cells. Optimal selection pressure will use the lowest level of toxin needed to kill the untransformed tissues.

Kuehnle and Sugii (1992) identified the potentially transformed tissues of *Dendrobium* by the growth and green colour on half-strength MS medium supplemented with 2% sucrose and 50-100 mgl^{-1} kanamycin sulphate. Kanamycin concentrations that prevented growth of non transformed tissues could not be used for long term selection because such levels suppressed the regeneration of potentially transformed tissues. Levels of 100-200 mgl^{-1} kanamycin were later found adequate for *Dendrobium* by Nan and Kuehnle (1995). Hsieh *et al.* (1997) obtained the transformant of *Phalaenopsis* from the proliferating proembryoids of the explant which were selected in regeneration medium containing 100 μg/ml kanamycin after 30 day culture period. Babu and Chawla (2000) transferred the explants of *Gladiolus*

to selection media with 100 mgl^{-1} kanamycin and 100 mgl^{-1} hygromycin depending upon the vector PBI141 and pTOK 233 used for co-cultivation. Thus the transformed plants are selected.

Potentially transformed tissues were identified by active growth on MS medium supplemented with 50 mg hygromycin/litre, for four to six months (Yu *et al.,* 1999). Belarmino and Mii (2000) used hygromycin at 50 mgl^{-1} concentrations to select the transformants.

Knapp *et al.* (2000) selected for transformed cells using bialaphos. PLBs which proliferated on selection medium containing 3 mgl^{-1} bialaphos were selected as transformants.

Kushikawa *et al.,* (2001) selected transformants in African violet after four months of subculture on the selection medium supplemented with 50 mgl^{-1} hygromycin B. Kim *et al.* (2002) selected the friable embryogenic callus and leaf with axil tissues on different hygromycin concentrations. As a result 20 mgl^{-1} hygromycin was identified as the best concentration to select both FEC lines and leaves with axil tissues. In *Phalaenopsis,* Chai *et al.* (2002) carried out selection on regeneration medium containing 3 mgl^{-1} hygromycin for two months.

Transgenic *Gerbera* plants were selected on medium with the maximum concentration (7 mgl^{-1}) of kanamycin at (Korbin *et al.,* 2002). In sedum (Yoon et *al.,* 2002) and carnation (Lin *et al.,* 2003) 25 mgl^{-1} kanamycin was used to select the transformants. Boase *et al.* (2002) selected the transformed cells with kanamycin 50 mgl^{-1}. The transgenic shoots produced were rooted in the presence of 100 mgl^{-1} kanamycin. Leaf disc explants were cultured on selection medium containing 100 mgl^{-1} kanamycin and 5 mgl^{-1} hygromycin or 300 mgl^{-1} kanamycin for selection and regeneration of transformants in *Begonia* (Kishimoto *et al.,* 2002).

In *Arabis gunnisoniana* the transgenic shoots were selected on MS medium supplemented with 50 mgl^{-1} kanamycin (Taskin *et al.*, 2003). The antibiotic concentrations used for selecting the transformants varied according to the cultivars in chrysanthemum (Seo *et al.*, 2003). They identified the suitable concentration of kanamycin to select the transformants in two different culivars. It was 20 mgl^{-1} kanamycin in Puma and 50 mgl^{-1} in Subangryeok. In Puma, the callus formation was most efficient in the medium containing kanamycin. Ram and Mohandas (2003) selected the transformants of African violet on the selection media containing kanamycin 70 mgl^{-1}.

Men *et al.* (2003) obtained putatively transformed plantlets by selection and regeneration on medium supplemented with 30 mgl^{-1} hygromycin. Chan *et al.* (2003) optimized the hygromycin concentration for selection of *Phalaenopsis* transformants. They reported that hygromycin 50 mg l^{-1} was the optimum.

2.4.11 Histochemical GUS Expression

Some reporter gene products can be detected in intact plant tissues. The most popular of these systems is the *E.coli* β-D-glucuronidase (GUS) gene. It encodes a stable enzyme that is not normally present in plants and that catalyzes the cleavage of a range of β-D-glucuronides. The GUS activity in transformed plant tissues can be localized by observing the blue colour that is formed after hydrolysis of the uncoloured substrate 5-bromo4-chloro3-indolyl β-D-glucuronic acid.

Nan and Kuehnle (1995) reported that in *Dendrobium,* tissue genotype and type of microparticle significantly affected transient GUS activity. Higher expression was seen in PLBs and in hybrid UH 44, compared to etiolated shoots and protocorms and to hybrids M61 and K1329-39. Nan and Kuehnle (1995) obtained on an average 10-16% transient GUS expression using PBI 121. Hsieh *et al.* (1997) observed the histochemical GUS activity in the transformed tissues of *Phalaenopsis.* A difference

in GUS activities occured among genotypes and proliferating stages of explants. The cultivar Brother Mirage A79-69 had the lowest frequency of GUS gene expression. True Lady A76-13 at the 10 to 12 days proliferating stage had 50-80% frequency of GUS expression. However, the highest frequency (100%) occurred at the 10 day proliferating stage of Asian Elegance B 79-11 and Taisuco Kaalatian F 80-13.

Steinhart *et al.* (1997) detected the transient expression of β-glucuronidase following the plasmid delivery into *Cattleya* protocorms and their protoplasts by particle bombardment. Transient expression was observed histochemically in the protocorms for up to three weeks. In *Alstroemeria* Cv VV024 the transformed friable embrogenic callus lines showed five times higher rate in transient GUS gene expression, than those of leaves with axil tissue (Kim *et al.*, 2002).

Belarmino and Mii (2000) confirmed the successful transformation in *Phalaenopsis* orchid by histochemical GUS assay. Kushikawa *et al.* (2001) observed in African violet that the cells transformed with *A. tumefaciens* strain LBA4404 (pTOK233) showed the highest *GUS* activity.

In *Sedum,* Yoon *et al.* (2002) reported that out of 640 co-cultivated leaf segments, 24 (3.75%) produced kanamycin resistant shoots. Out of the total, 2.5 per cent shoots were *GUS* positive. Among the *GUS* positives 94 per cent were transferred to soil and they produced flowers. In *Cyclamen* Boase *et al.* (2002) observed that 112 transformed shoots were produced and they were rooted in the presence of 100 mg l^{-1} kanamycin. Out of them 47 per cent were positive for *GUS.*

Leaves, roots and some newly formed PLBs were taken from hygromycin (3 mg l^{-1}) resistant rooted plantlets of *Phalaenopsis* and then subjected to *GUS* assay. Newly formed PLBs from GUS positive plants were regenerated on the regeneration medium containing a lower concentration of hygromycin (1.5 mg/l). When the PLBs regenerated to plantlets, the original PLBs were subjected to GUS assay. This

procedure was repeated monthly for four cycles and it was found that a long selection period with low hygromycin concentration yielded stable transformants. Histochemical *GUS* assay indicated successful transformation (Chai *et al.*, 2002)

Around 72.1 per cent of the transformed carnation plants rooted in the medium containing kanamycin at 25 mg l^{-1}. Around 55 per cent of the plants showed *GUS* activity (Lin *et al.*, 2003). Liau *et al.* (2003) obtained 28 independent transgenic *Oncidiums* from which six were positive for β-glucuronidase.

Chen *et al.* (2002) observed 100% *GUS* expression. *GUS* expression fell off sharply as the infection time and duration of co-culture increase. *GUS* gene was detected even from the new rhizome as well as from the shoots derived from a rhizome in which the *GUS* gene had been introduced. Younger rhizomes had strong *GUS* expression. Although transmission of the *GUS* gene in the original transgenic explant to the next subculture explants was almost 100% in the first sub culture, the expression decreased in subsequent subcultures. Successful transformation was confirmed by *GUS* histochemical assay.

Transient *GUS* expression was the highest when *Rhododendron,* PJM hybrid leaf explants were incubated on the regeneration medium 9-12 days before bombardment. Transient *GUS* expression by Iridon *Chrysanthemum* leaf explants was relatively high (>42%) for leaves incubated 3-18 days before bombardment. Exposure of *Rhododendron* leaf explants to media containing different concentrations of sucrose decreased transient *GUS* expression by 71 per cent, compared to those on sucrose free medium. In *Chrysanthemum,* 27 per cent more explants transiently expressed *GUS,* compared to those on sucrose free medium and the number of spots per *GUS* positive explant increased from five to thirteen. Transient *GUS* expression increased almost three fold when the *Rhododendron* leaf explants were placed in the dark for six days, compared to those in the light. In contrast, the dark treatment had

inconsistent effects on transient *GUS* expression by chrysanthemum Iridon explants (Moore and Tripepi, 2003).

2.4.12. PCR Analysis

PCR analysis is used to analyse the presence of transgene in the genome of the transformed plant. PCR analysis of the transgenic plants of *Dendrobium* Jaquelyn Thomas showed that eight of the nine regenerated UG 800 plantlets contained both *neo* and *gus A* fragments. This suggested that the plasmid DNA may be fragmented or rearranged during this procedure (Kuchnle and Sugii, 1992). Similar observations were also reported in *Dendrobium* White Angel (Chia *et al.,* 1994). Griesbach (1994) reported the GUS activity in 3 to 6 week old protocorms and in one year old seedlings of *Calanthe.* In this atleast two plants were positive for *gus A* by PCR analysis, one year after the treatment. In *Phalaenopsis,* Belarmino and Mii (2000), and Chai *et al.* (2002) confirmed the presence of transgene by PCR analysis.

Presence of *bar* gene in the transformed plants of *Brassia, Cattleya* and *Doritaenopsis* was confirmed by PCR analysis (Knapp *et al.,* 2000). In *Dendrobium* the presence of transgene was assessed by PCR analysis (Yu *et al.,* 2001). Mishiba *et al.* (2000) supported the transgenic nature of regenerated plants of lavender by PCR analysis of the DNA of putative transgenic plants obtained. The 1.2 kb and 0.7 kb fragments which were expected to be amplified by primers for introduced *gus* and *npt* genes, respectively, were observed in each transgenic plant. No amplification of the expected band was observed in the non-transformed control plant. Successful transformation in *Cymbidium* was confirmed by PCR analysis of transformants (Chen Li *et al.,* 2002). In cape daisy, transformation with lettuce mosaic virus derived constructs was confirmed by PCR (Morbel *et al.,* 2002). The presence of the transgene in gerbera genome was confirmed by PCR (Korbin *et al.*, 2002).

3. MATERIALS AND METHODS

The experiments on *Agrobacterium* mediated genetic transformation in *Dendrobium* were carried out at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani during January 2002 to October 2004. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 SOURCE OF EXPLANT

Seeds were collected from the pods of *Dendrobium,* Sonia 17 at three-fourth maturity stage. Protocorms produced from the germinating seeds were used as explants for the experiment. Protocorm is a small storage organ formed from the germinating embryo, possessing an apical meristem and a leaf primordium.

Protocorm like bodies (PLBs) were established by culturing two-year old shoot apices of *Dendrobium.* The shoot apices were collected, trimmed and subjected to various surface sterilization treatments with mercuric chloride (0.08 and 0.1 per cent). The *in vitro* raised PLBs were also used as the source explant for the study. PLBs are somatic protocorms derived from *in vitro* culture of apical or axillary bud meristems. Primary PLBs are induced by culturing apical meristem tips. Secondary PLBs are the PLBs formed on the surface of a primary PLB. Proliferate PLBs are the PLBs proliferating on the surface of either primary or secondary PLBs

3.2. CULTURE MEDIUM

3.2.1. Chemicals

All the chemicals used for the preparation of the culture media were of analytical grade and procured from Sisco Research Laboratories (SRL), India. The antibiotics and plant growth substances were obtained from Himedia Laboratories, Mumbai.

3.2.2. Glassware

Borosilicate glassware of Borosil brand and disposable sterile petridishes of Tarson's and membrane filters from Sartores, Germany were used for the experiments.

3.2.3. Composition of Media

Basal MS medium (Murashige and Skoog, 1962) and modifications of the media supplemented with various plant growth substances and organic supplements, were used for plant tissue culture experiments. Yeast Extract Peptone (YEP) medium (An *et al*., 1988). Yeast Extract Maltose (YEM) medium, Luria Burtani (LB) medium and AB medium were used for bacterial culture during the study. The composition of these media is given in Appendix I and II.

3.2.4. Preparation of Medium

Standard procedures (Murashige and Skoog, 1962) were followed for the preparation of the plant tissue culture media. After mixing appropriate quantities of the stock solutions and making up the volume to the required quantity by using double distilled water, the pH of the medium was adjusted to 5.7 using 0.1N NaoH/HCl. Agar was then added at the rate of 6-7g 1^{-1} and the medium was heated to mix agar. Then the medium was dispensed to the culture vessels at the rate of 15ml/culture tube, and 25ml/petriplate. The test tubes were plugged with non-absorbent cotton. Autoclaving was done at 121°C and 1.06 kg/cm^2 pressure for 20 minutes (Dodds and Roberts, 1982) to sterilize the medium. Activated charcoal was added to the medium, and it was shaken gently before solidification, for uniform distribution of charcoal throughout the media. The medium was allowed to cool to room temperature and stored in culture room until used.

For bacterial culture, the pH of the medium was adjusted to 7.0. Solidification of the medium was done using agar at the rate $20gl^{-1}$. The medium was sterlized by autoclaving for 20 minutes at $1.06kg/cm^2$ and 121°C. The medium was prepared in conical flasks and stored in culture room.

3.3. TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic measures were carried out under the hood of a clean laminar air flow cabinet (Thermodyne) fitted with UV lamp.

3.4 CULTURE CONDITIONS

The cultures were incubated at 26°C in an air-conditioned culture room with 16h photoperiod (1000 lux) supplied by cool white fluorescent tubes. Humidity in the culture room varied between 60 and 80 per cent.

3.5 PRODUCTION OF PROTOCORMS

The protocorms were obtained by the germination of *Dendrobium* capsules. Capsules of *Dendrobium* were collected, dipped in water containing a few drops of Labolin, for 30 minutes and rinsed four to five times with water. The capsules were taken to the laminar airflow cabinet and surface sterilized with $HgCl_2$ (0.08 and 0.1%) for ten minutes. They were washed free of $HgCl_2$ by rinsing with five changes of sterilized distilled water. Then the capsules were dipped in alcohol and flamed in a bunsen burner. They were then cut open and the yellow seeds were scooped out. The seeds were inoculated on half-strength MS medium supplemented with different doses of coconut water, sucrose $30gl^{-1}$ and agar $6.0gl^{-1}$. The cultures were incubated in the culture room, in darkness. Observations on the germination per cent and time taken for germination, were recorded.

3.6 ESTABLISHMENT OF PROTOCORM LIKE BODIES (PLBS)

Primary protocorm like bodies were induced by culturing shoots apices. The shoots were collected and the shoot apices were cut out and washed in running tap water, followed by washing in water containing a few drops of Labolin and rinsed with four or five changes of water. Then the explants were trimmed using blade and taken to the laminar airflow chamber and surface sterilized with $HgCl_2$ (0.08 and 0.1%) for ten minutes. They were then washed free of $HgCl_2$ by rinsing with four changes of sterile distilled water. Then the explants were transfered to sterile filter paper for absorbing the excess moisture. The meristems were inoculated in culture bottles containing half-strength MS semisolid medium supplemented with sucrose $30gl^{-1}$, agar $6.0gl^{-1}$, different combinations of organic supplements, and growth substances *viz.*, NAA and BA. The cultures were incubated at 26°C in the culture room with 16h photoperiod (1000 lux). Observations were recorded on the number of primary PLBs, secondary and proliferating PLBs formed on the surface of the primary PLBs.

3.6.1. Proliferation of Protocorm Like Bodies (PLBs)

The primary and secondary PLBs formed were cut and divided into individual PLBs using sterile scalpel blade and cultured in bottles. The cultures were maintained in the culture room at 26°C. The cultures were changed to fresh media before the PLB started to form shoots. Observations were recorded on the number of PLBs formed at weekly intervals.

3.7 EVALUATION OF ORCHID CULTURES FOR SENSITIVITY TO ANTIBIOTICS

Sensitivity of orchid cultures to antibiotics was evaluated to utilize it as a marker system for selection purposes. Both protocorms and PLBs were tested for

their sensitivity to various antibiotics *viz.,* ampicillin, rifampicin, carbenicillin, cefotaxime, kanamycin and hygromycin at varying concentrations ranging from 5 to 500 mgl^{-1}. Both protocorms and PLBs were pre-cultured for a period of 15 days and the newly formed tissues were used.

3.7.1 Ampicillin

The PLBs proliferation medium, *viz.,* half-strength MS supplemented with coconut water $150mll^{-1}$, activated charcoal $0.5gl^{-1}$, sucrose $30gl^{-1}$, agar $6.0gl^{-1}$ and BA $0.2mgl^{-1}$ was used for the PLBs. For testing the protocorms, half-strength MS medium supplemented with coconut water $150mll^{-1}$, sucrose 30 gl^{-1} and agar $6.0gl^{-1}$ was used. The medium was prepared in conical flasks of suitable size and stored in culture room until use.

On the day of the experiment, the medium was melted and then cooled to a temperature of 40°C. Ampicillin stock was prepared by dissolving ampicillin in water. The melted and cooled medium, and the ampicillin stock were taken to the laminar airflow cabinet. The required antibiotic solutions at various concentrations ranging from 5 to 500 mgl^{-1} were diluted from the stock, filter-sterlized and added to the medium as per the treatments and mixed thoroughly by gentle swirling. The gentle swirling avoided the production of air bubbles. The medium containing the antibiotic was dispensed to sterilised empty petridishes (90 mm) and allowed to cool and solidify. When the medium was cooled to room temperature and solidified, the explants *viz.*, protocorms and protocorm like bodies (PLBs) were inoculated. The petridishes were sealed with parafilm and kept in the culture room.

The culture response of the explants to different antibiotic concentrations was evaluated at periodic intervals (weekly) based on scoring as follows:

Score	Culture Response
'++++'	Fully green
'+++'	Partial discoloured
'++'	Bleached tissues
'+'	Tissues turning brown and dead

3.7.2. Rifampicin

Ethanol/methanol was used to dissolve rifampicin. The stock solution of rifampicin was prepared by dissolving it in sterile water. On the day of the experiment the medium was melted and allowed to cool to 40°C before adding the antibiotics. The antibiotic solutions at varying concentrations ranging from 5 to 500 mgl^{-1} were prepared fresh from the stock, filter sterilised and added to the cooled medium before it solidified. In order to avoid the production of air bubbles, the medium with the antibiotics was swirled. The medium from the conical flask was dispensed to sterile petridishes (90 mm). Sweating of the plates was avoided by keeping the petriplate lids half opened in the laminar airflow cabinet. When the medium cooled down, and solidified, the explants *viz.*, protocorms and PLBs were inoculated. The petridishes sealed with parafilm and kept in the culture room. The culture response of the explants was scored at weekly intervals.

3.7.3. Cefotaxime

The stock solution of cefotaxime was prepared by dissolving it in sterile water. On the day of the experiment the medium was melted and allowed to cool to 40°C before adding the antibiotics. Then antibiotic solutions at varying concentrations ranging from 5 to 500 mgl^{-1} were prepared fresh from the stock, filter sterilised and added to the cooled medium before it solidified. In order to avoid the production of air bubbles, the medium with the antibiotics was swirled. The medium from the conical flask was dispensed to sterile petridishes (90 mm). Sweating of the plates was avoided by keeping the petriplate lids half opened in the laminar airflow cabinet. When the medium cooled down, and solidified, the explants *viz.*, protocorms and PLBs were inoculated. The petridishes sealed with parafilm and kept in the culture room. The culture response of the explants was scored at weekly intervals.

3.7.4. Carbenicillin

Carbenicillin stock solution was prepared by dissolving disodium carbenicillin in water. The antibiotic solutions at varying concentrations ranging from 5 - 500 mgl^{-1} were prepared fresh from the stock, filter-sterilised and added to the cooled medium before it solidified. Then the medium was poured in sterile petridishes. When the medium in the petridish cooled down and solidified, the explants were inoculated and the petriplates sealed and kept in culture room. The culture response of these explants was scored at weekly intervals.

3.7.5. Kanamycin

Kanamycin stock solution was prepared by dissolving kanamycin in water. The antibiotic solutions at varying concentrations ranging from 5 to 500 mgl^{-1} were prepared fresh from the stock, filter-sterilised and added to the cooled medium before it solidified. Then the medium was poured in sterile petridishes. When the medium

in the petridish cooled down and solidified, the explants were inoculated and the petriplates were sealed and kept in culture room. The culture response of these explants was scored at weekly intervals.

3.7.6. Hygromycin

Hygromycin stock solution was prepared by dissolving hygromycin in water. The antibiotic solutions at varying concentrations ranging from 5 to 500 mgl^{-1} were prepared fresh from the stock, filter-sterilised and added to the cooled medium before it solidified. Then the medium was poured in sterile petridishes. When the medium in the petridish cooled down and solidified, the explants were inoculated and the petriplates sealed and kept in culture room. The culture response of these explants was scored at weekly intervals.

3.8. BACTERIAL STRAINS AND BINARY VECTORS

Two strains of *Agrobacterium tumefaciens viz.,* LBA 4404 and EHA 105 were used for the study. EHA 105 by virtue of harbouring the 'supervirulent' *'vir'* gene, exhibit broader host range and higher transformation efficiency. The bacterial kanamycin reistance gene in EHA 101 was deleted to develop the *'vir'* helper strain EHA 105.

Two *Agrobacterium* strains with three different binary vectors were used for the study.

1. PBI121 (Chen *et al.,* 2003)

The T-DNA of PBI 121 contain the *gusA* reporter gene under the control of CaMV35S promoter and the selectable marker gene *nptII*

2. pCAMBIA 1301 (Hajdukiewicz *et al.*, 1994)

The T DNA of pCAMBIA 1301 harbours the gene *gus A*. The MCS (matrix coding sequence) is between *gus A* (proximal to the right T DNA border) and hygromycin resistance gene *hpt II (*proximal to the left T-DNA border). These two genes are under the control of CaMV35S promoter.

3. pCAMBIA 2301 (Hajdukiewicz *et al.*, 1994)

The T-DNA of this binary vector contains the *gus A* gene and the *npt II* (Kanamycin resistance) gene under the control of CaMV35S promoter.

The pPZP vector backbone (A new series, small and stable) was used to construct the pCAMBIA series of vectors with *npt II, hpt* and *bar* as selection markers and *gus* or *gfp* as reporters (Veluthambi *et al.,* 2003). The pCAMBIA vectors are widely used for transformation experiments. One other important element that has been used is the promoter for the 35S RNA from cauliflower mosaic virus (CaMV35S Promoter). In most systems this promoter is constitutively expressed and it is 30-50 times stronger than the Nos promoter. This permits higher level of expression of the gene of interest.

3.8.1. Culturing of Bacteria with the Vectors

The *Agrobacterium tumefaciens* strains LBA4404 and EHA105 harbouring different binary vectors were cultured on plates with YEP, YEM and AB medium. The time taken for the growth of the different strains with the vectors was observed.

3.9. SCREENING OF *AGROBACTERIUM* STRAINS FOR SENSITIVITY TO ANTIBIOTICS

The YEP medium was prepared in required volume in conical flasks and kept in the culture room until use. On the day of the experiment the medium was melted and then cooled to 40°C.

3.9.1. Ampicillin

Ampicillin stock was prepared by dissolving ampicillin in water. When the YEP medium was cooled and solidified the bacterial strains with the vectors were spread on the medium with an L rod and then the petriplates sealed with a parafilm and kept in the culture room. The growth of different bacterial strains was recorded.

3.9.2. Rifampicin

Ethanol/methanol was used to dissolve rifampicin. The stock solution of rifampicin was prepared by dissolving it in sterile water. When the melted YEP medium cooled down, and solidified, the bacterial strains with the vectors were spread on the medium with an L rod and then the petriplates sealed with a parafilm and kept in the culture room. The growth of different bacterial strains was recorded.

3.9.3.Cefotaxime

The stock solution of cefotaxime was prepared by dissolving it in sterile water. When the melted YEP medium cooled down, and solidified, the bacterial strains with the vectors were spread on the medium with an L rod as in **3.9.2.** and then the petriplates sealed with a parafilm and kept in the culture room. The growth of different bacterial strains was recorded.

3.9.4. Carbenicillin

Carbenicillin stock solution was prepared by dissolving disodium carbenicillin in water. The antibiotic solutions at varying concentrations ranging from 5 to 500 mgl^{-1} were prepared; filter-sterilised and added to the cooled medium before it solidified. Then the medium was poured in sterile petridishes. When the medium in the petridish cooled down and solidified, the bacterial strains with the vectors were spread on the medium with an L rod. The sealed with a parafilm and kept in the culture room. The growth of different bacterial strains was recorded.

3.9.5. Kanamycin

Kanamycin stock solution was prepared by dissolving kanamycin in water. To the melted and cooled YEP medium the bacteria was spread as in **3.9.2.** then the petriplates sealed with a parafilm and kept in the culture room. The growth of different bacterial strains was recorded.

3.9.6. Hygromycin

Hygromycin stock solution was prepared by dissolving hygromycin in water. When the medium in the petridish cooled down and solidified, the bacterial strains with the vectors were spread on the medium with an L rod as in **3.9.2.** Then the petriplates were sealed with a parafilm and kept in the culture room. The growth response of different bacterial strains was recorded.

3.9.7. Test for Bacteriocidal Activity of Cefotaxime

Twenty-five ml of the regeneration media of protocorms (half-strength MS media supplemented with coconut water 150mll^{-1} and sucrose 30gl^{-1}) and PLB proliferation medium (half-strength MS supplemented with coconut water 150mll^{-1}, activated charcoal 0.5gl^{-1}, sucrose 30gl^{-1}, and BA 0.2 mgl^{-1}) were taken in 100ml

conical flasks. To these 5.0 ml of the bacterial suspension raised in YEP medium with an O.D. of 0.9 was added and incubated in shaker (rpm 110) at 28°C for 48 hours. All the cultures developed an O.D. of 1.1. Cefotaxime at concentrations ranging from 100 to 500 mgl^{-1} was added to these cultures. One flask was kept without the addition of cefotaxime. These cultures were again incubated in shaker (rpm 110) at 28°C for 48 hours. These suspension were spread on petriplates with L rod, and the petriplates sealed and kept in the culture room. Observation on bacterial growth were recorded.

3.10. PRODUCTION OF *AGROBACTERIUM* TRANSFORMANTS BY TRIPARENTAL MATING

The binary plasmid was transferred to *Agrobacterium* by a triparental mating process. This mating was facilitated by a conjugative plasmid PRK 2013.

On the first day *Agrobacterium tumefaciens* was streaked on plates with AB minimal medium supplemented with 20 µg/ml rifampicin. This was kept for growth at 28^0C for 48 hrs. On the next day *E.Coli* strains (PRK 2013) and (PPE 2113) were streaked on plates with LB medium supplemented with kanamycin 50 µg/ml. On the third day triparental mating was performed by mixing all these three strains (Two *E-coli* strains one with the binary vector, the other with helper plasmid, PRK 2013; and the *Agrobacterium* strain). This mating was done in AB minimal plate without antibiotics. This plate was incubated at 28°C for 48 hours. Then the transformants were selected by streaking the mixture of strains obtained by triparental mating on AB minimal plate containing rifampicin (20 µg/ml) and kanamycin 50 µg/ml. Simultaneously two control plates were also streaked with AB minimal and rifampicin (20 µg/ml) and AB minimal plate with kanamycin 50 µg/ml. These two plates were kept at 28^0C for three days. No growth was observed in these two control plates. On those plates with rifampicin (20 µg/ml) and kanamycin (50 µg/ml), the

transformed *Agrobacterium* survived. A single colony was picked up for further experiments.

3.11. GENETIC TRANSFORMATION OF *DENDROBIUM*

3.11.1. Preparation of *Agrobacterium* Suspension for Co-cultivation

The two *Agrobacterium* strains with the three binary vectors were grown on petriplates with AB medium. *Agrobacterium* suspension for co-cultivation was prepared by picking a single colony from a plate. This was inoculated in 20 ml liquid medium containing the required antibiotics and 50, 100, 150, 200 μM acetosyringone. The liquid medium was kept in a shaker overnight. Then the bacterial suspension was spun in a centrifuge at 5000 rpm at 4°C for 5 minutes. The pellets obtained were resuspended in half-strength liquid MS medium, with 50, 100, 150, 200 μM acetosyringone. The overnight grown culture was also diluted to 1:5 (v/v) with the fresh medium and the optical density (OD) of the culture was measured at A 600 nm and the bacterial concentration was adjusted to 0.1 by dilution or concentration.

3.11.2. Preparation of Plant Material

Protocorms and PLBs were used as explants for the co-cultivation experiments. These explants were precultured fifteen days before co-cultivation to initiate new growth and to be in active cell division. Explants of different sizes, protocorms (0.1, 0.2 cm) and the PLBs (0.1, 0.2, 0.3, 0.4, 0.5 cm) were co-cultivated to standardise the optimum size for maximum transformation efficiency. The optimum number of explants (20, 30, 40, 50) that should be kept in a single petridish (90 mm) during co-cultivation was standardised, by co-culturing (20, 30, 40, 50) explants in a single petriplate.

3.11.3. Co-cultivation

The precultured protocorms and PLBs were used for the co-cultivation experiments. They were cut by sterile knives and wounded to facilitate the infection process. The initial step in the infection process is the attachment of *A. tumefaciens* to a plant cell at the site of an open wound. *A. tumefaciens* responds to certain phenolic compounds such as acetosyringone and hydroxyacetosyringone, which are exuded by wounded plants. These small molecules act to induce the activity of virulence *(vir)* genes that are encoded on the plasmid.

For co-cultivation the explant pieces were placed in a sterile petridish kept in a laminar airflow cabinet. The drying of explants was avoided by wetting them with liquid half strength MS medium. Then the explants were mixed thoroughly with the prepared *Agrobacterium* suspension and acetosyringone (50, 100, 150, 200 μM) by gentle swirling. This gentle swirling was done for 10, 15 and 20 minutes so as to standardise the optimum time required for the infection process.

3.11.4. Addition of Acetosyringone

After mixing, the explants were blotted with sterile filter paper and placed in a petriplate containing half-strength MS medium and acetosyringone at concentrations (50, 100, 150, 200 μM) which ranged according to the treatments. Explants were blotted and kept in petriplate without blotting to standardise the blotting requirements. Then the petriplates were sealed with parafilm and bacteria with the binary vectors and the plant tissues were co-cultivated in dark for two, three, four and five days at 26°C in a culture room. The effect of number of days of co-cultivation on maximum transformation efficiency was standardised.

3.11.5. Incubation on Non-selection Medium

After co-cultivation, the tissues were washed in half-strength liquid MS medium containing 200 mgl^{-1} cefotaxime. They were blotted dry with sterile filter paper. Then they were transfered to sterile petriplates containing half- strength MS medium with 200 mgl^{-1} cefotaxime for eradication of excess *Agrobacterium.*

3.11.6. Selection of Transformed tissues

The transformed tissues were selected on half-strength MS medium containing kanamycin 200 mgl^{-1} (for the tissues cocultivated with vectors PBI 121, and pCAMBIA 2301) and hygromycin 100 mgl^{-1} (for the tissues transformed with pCAMBIA 1301). The bacteriostatic agent cefotaxime was also added in the initial subcultures. The tissues were maintained by subculturing once in ten days. In eight weeks, the transformed tissues developed green sprouts along the cut edges. The control plants on the same culture media bleached. The kanamycin concentration was gradually increased upto 400 mgl^{-1} and the bacteriostatic agent is removed after the third subculture. The transformed tissues were grown in medium containing antibiotic for a period of eight months.

3.12. GUS HISTOCHEMICAL ASSAY

A histochemical assay (Jefferson *et al.,* 1987) was used to detect the expression of the *gus* gene (B - glucuronidase). Transient expression of the GUS gene was visualized 24 to 30 hours after staining the explants for GUS activity. The transformed tissues were incubated in GUS substrate X-gluc, for 24 hours at 37°C in the dark. The X-gluc solution was prepared by mixing 10 mg X-gluc in 100 µl dimethyl formamide, 50 mM sodium phosphate buffer, 0.1% (v/v) Triton X 100. X-gluc is a well-established chromogenic substrate used for measuring *gus* activity. The enzyme converts this substrate to an insoluable, intense indigo-blue

chromophore. Gus expressing cells were detected as blue spots on the explant under a microscope (Nikon, smz-10A, Type 115). Each spot was scored as one transformation event, regardless of its size (Puddephat *et al.,* 1999).

3.13. PCR ANALYSIS

3.13.1. DNA Extraction

The extraction protocol was modified from that of Mondal *et al.* (2000) with out the use of cTAB. Leaf material (0.5 g) was pulverized in liquid nitrogen with 20.0 (μlβ - mercaptaethanol in a pre-cooled mortar by rapid grinding to a fine power. Then 7.5 ml of hot (65°C) extraction buffer (100 mM Tris – Hcl, 20 mM EDTA, 2 M NaCl, 2 per cent (w/v) SDS, pH = 8) and a pinch of polyvinyl pyrrollidone (PVP) were added. The fine slurry of grounded plant material was transfered to a 50 ml conical flask and incubated in water bath at 65°C for 20 minutes with occasional gentle shaking. The lysate was then squeezed through four layers of sterile muslin cloth into a sterile centrifuge tube. After that an equal volume of chloroform: Isoamyl alcohol (24:1) was added and thoroughly mixed. The mixture was centrifuged at 1000 rpm for 10 minutes at 20°C. The supernatant was transferred to another sterile centrifuge tube with a wide bore sterile pipette tip. To this again equal volume of chloroform : Isoamyl alcohol (24 : 1) was added and centrifuged as in the previous step after thorough mixing. After that to the supernatant, $1/10^{th}$ volume of chilled absolute alcohol was added. It was mixed gently and then centrifuged at 10,000 rpm for 10 minutes at 4°C to pellet the DNA. The supernatant was discarded and the pellet was washed in 70 per cent ethanol. The pellet was air dried and then dissolved in 0.5 ml of 1X Tris and EDTA buffer (10mM Tris HCl, 1mM EDTA, p^H 8) stored at 4°C.

3.13.2 Quantification of DNA

Quantification of DNA is necessary before it is subjected to amplification. Quantification of DNA was carried out with the help of UV – Vis spectrophotometer (Spectronic Genesys 5).

The buffer in which the DNA was already dissolved was taken in a cuvette to calibrate the spectrophotometer at 260 and 280 nm wavelengths. The optical density (OD) of the DNA samples dissolved in the buffer was recorded at both 260 and 280 nm. The concentration of DNA was found out using the formula.

Amount of DNA (ng/μl) = A_{260} x 50 x dilution factor / 1000

Where A_{260} – absorbance at 260 nm

The quality of the DNA could be judged from the ratio of the OD values recorded at 260 nm and 280 nm. The A_{260}/A_{280} ratio between 1.8 and 2.0 indicates best quality of DNA, where A_{280} is the absorbance at 280 nm.

3.13.3 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit. Required amount of agarose was weighed out (1.0 per cent) and melted in 1 x TAE buffer (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0) by boiling. After cooling to about 50°C, ethidium bromide was added. The mixture was then poured to a present template with appropriate comb. After solidification of agar, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank. The tank was loaded with 1 x TAE buffer, so that it jsut covered the entire gel. Required volume of DNA sample and gel - loading buffer (6.0 x loading dye viz., 40 per cent sucrose, 0.25 per cent bromophenol blue) were mixed. Each well was loaded with 20 μl of sample. One of the wells was loaded with 5.0 μl of PCR

molecular weight marker along with required volume of the gel loading buffer. Electrophoresis was performed at 75 volts until the loading dye reached 3/4th of the length of the gel. The gel was visualized using an ultraviolet visible (UV - Vis) transilluminator.

3.14. Statistical analysis

The collected data were subjected to the analysis of variance to test the significance of the difference among the treatment means.

4. RESULTS

The results of the experiments on *Agrobacterium* mediated genetic transformation techniques in *Dendrobium* Sonia 17 carried out in the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani during January 2002 to October 2004 are presented below.

4.1. ESTABLISHMENT OF *IN VITRO* CULTURE

4.1.1. *In vitro* Establishment of Protocorms

4.1.1.1. Surface Sterilization

The culture establishment with 0.08 per cent mercuric chloride was 80.8 per cent while it was 45.9 per cent with 0.1 per cent mercuric chloride (Table 1). After 0.08 per cent mercuric chloride treatment for 10 minutes, flaming the pods in burner after dipping in 70 per cent ethanol was found to be effective. The highest establishment percentage (84.5) was obtained with two stroke flaming treatment.

4.1.1.2. Effect of Coconut Water on in vitro Germination and Protocorm Initiation

Effect of different doses of coconut water on germination and number of days taken for protocorm initiation was studied (Table 2). Out of the six treatment with coconut water, CW 150 ml l^{-1} was found to be the best in terms of per cent germination (84.84) and early initiation of protocorms (50.62 days). All the treatments with coconut water resulted in early initiation and better germination compared to the control.

Table 1 Effect of pod sterilisation treatments on *in vitro* establishment of protocorm

S.No.	Sterilant	Concentration (%)	Per cent establishment
1.	Mercuric chloride	0.08	80.80
2.	Mercuric chloride	0.10	45.90
3.	Flaming	One stroke	78.80
4.	Flaming	Two strokes	84.50

Table 2 Effect of coconut water on germination and time taken for protocorm initiation in *Dendrobium*

Medium : MS (½ strength) + sucrose 30 gl^{-1} + agar 6g l^{-1}

S.No.	Concentration of coconut water ($ml\ l^{-1}$)	Per cent germination	Time taken for Protocorm initiation (days)
1.	100	82.03	54.30
2.	150	84.84	50.62
3.	200	83.45	50.60
4.	250	78.67	49.97
5.	300	77.50	49.50
6.	Nil	70.00	66.20
	SE_m CD (0.05)	2.19 6.76	1.50 4.65

4.1.1.3. Effect of Charcoal on in vitro Germination and Protocorm Initiation

There was significant difference on the germination percentage with various concentration of charcoal (Table 3). The highest germination (72.35%) was recorded with the addition of charcoal at 0.5 g l^{-1}. But increasing the concentration of charcoal to 1.0, 1.5, 2.0 g l^{-1} resulted in lesser germination percentage.

Significant difference could be observed with respect to the time taken for protocorm initiation. Early initiation of protocorms (65.18 days) was observed in 0.5 g l^{-1} which was on par with the control (66.20). The initiation of protocorms was found to slow down with the progressive increase in the concentration of charcoal from 1.0 to 2.0 g l^{-1}.

4.1.2. *In vitro* Establishment of PLBs

4.1.2.1. Surface Sterilization

Surface sterilization of the shoot tips with 0.10 per cent mercuric chloride for ten minutes was the most effective treatment (Table 4). The culture establishment with 0.1 per cent mercuric chloride treatment was 96.33 per cent, while the percentage establishment with 0.08 per cent mercuric chloride was 75.68.

4.1.2.2. Effect of Coconut Water on in vitro Proliferation of PLBs

There was significant difference on the percentage establishment and the proliferation rate of the PLBs with different concentrations of coconut water (Table 5). The maximum establishment percentage was observed with coconut water 150 ml l^{-1} (95.0%). The control recorded only 66.7 percentage.

Table 3 Effect of charcoal on in *vitro* seed germination and protocorm initiation, in *Dendrobium*

Medium : MS (½ Strength) + sucrose 30g l^{-1} + agar 6g l^{-1} + CW 150ml l^{-1}

S.No.	Concentration of Charcoal (gl $^{-1}$)	Per cent germination	Time taken for protocorm initiation
1.	0.5	72.35	65.18
2.	1.0	71.95	68.90
3.	1.5	71.09	70.50
4.	2.0	70.92	72.15
5.	Nil	70.0	66.20
	SE_m	2.42	1.48
	CD (0.05)	7.29	4.47

Table 4 Effect of surface sterilization on the establishment of shoot tip cultures

Sl.No.	Sterilant	Concentration (%)	Per cent establishment
1.	Mercuric chloride	0.08	75.68
2.	Mercuric chloride	0.10	96.33

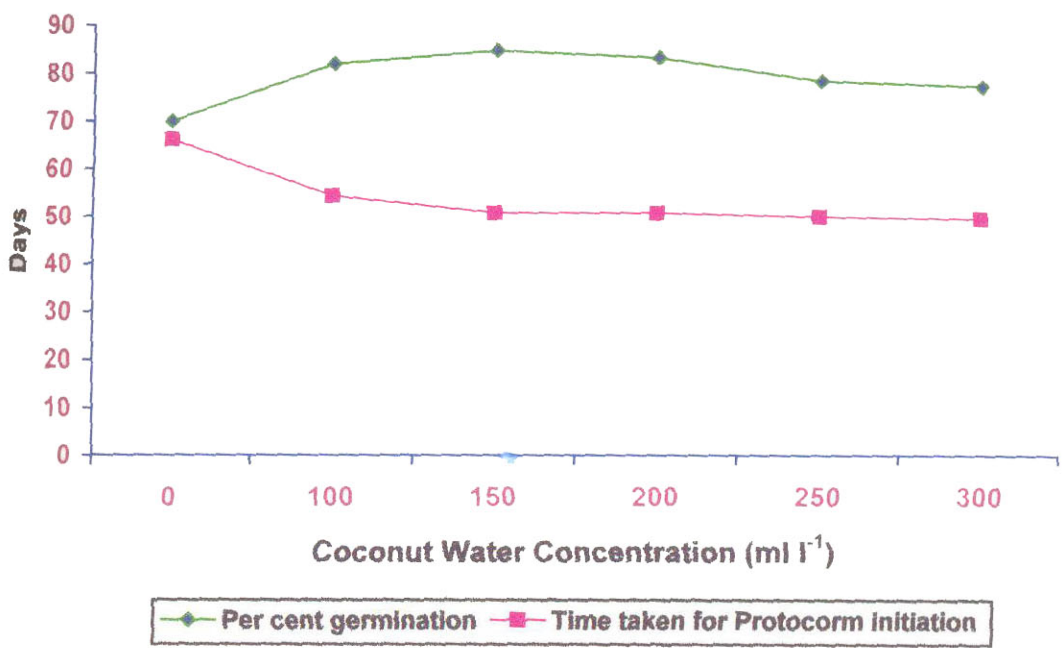


Figure 1 Effect of coconut water on germination and time taken for protocorm initiation in *Dendrobium*

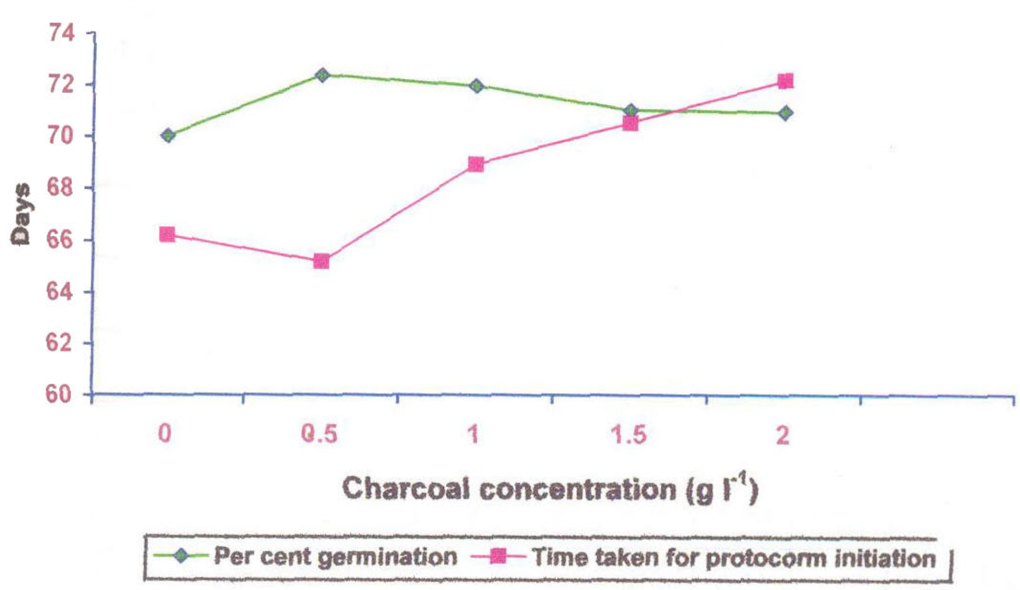


Figure 2 : Effect of charcoal on *in vitro* seed germination and protocorm initiation, in *Dendrobium*

Table 5 Effect of coconut water on *in vitro* proliferation of the protocorm like bodies

Medium: MS (½ strength) + sucrose 30g l^{-1} + agar 6g l^{-1}

S.No.	Coconut water ml l^{-1}	Percent establishment	Proliferation rate of PLBs (weeks)							
			I	II	III	IV	V	VI	VII	VIII
1.	100	83.30	3.30	5.67	7.69	9.33	11.20	13.60	5.70	17.80
2.	150	95.00	4.00	8.25	12.50	16.0	18.50	21.20	24.20	26.01
3.	200	82.30	3.60	6.60	9.25	11.67	13.70	16.50	18.20	20.50
4.	250	80.50	3.60	7.67	10.10	13.58	15.50	17.20	19.50	21.30
5.	300	70.20	3.50	8.00	11.25	14.33	16.20	18.40	20.00	22.40
6.	Nil	66.70	3.00	5.00	5.60	6.30	8.30	10.20	12.50	14.70
	$S.E_m$ C.D (0.05)	2.15 6.40	0.30 0.88	0.25 0.75	0.43 1.29	0.46 1.38	0.34 0.99	1.10 3.26	1.11 3.29	0.94 2.81

Table 6 Effect of activated charcoal on *in vitro* proliferation of the protocorm like bodies

Medium: MS (½ strength) + sucrose 30gl^{-1} + CW 150 ml l^{-1}+ agar 6gl^{-1}

S. No.	Concentation of charcoal (g l^{-1})	Percent establish ment	Proliferation rate of PLBs (Weeks)							
			1	2	3	4	5	6	7	8
1.	0.5	87.50	4.20	8.70	13.7	16.3	19.20	21.90	24.70	27.90
2.	1.0	75.00	3.80	8.25	12.8	15.8	18.70	19.50	21.20	24.50
3.	1.5	72.03	3.70	5.50	8.25	11.3	14.60	15.20	17.80	19.80
4.	2.0	70.10	3.50	4.20	7.00	9.50	12.80	13.90	15.50	16.20
5.	Nil	62.50	3.30	5.35	5.73	6.70	8.50	10.50	13.10	15.50
	SE_m C.D (0.05)	1.07 3.24	0.26 0.80	0.22 0.67	0.41 1.24	0.45 1.38	0.63 1.89	0.40 1.20	1.01 3.05	0.82 2.47

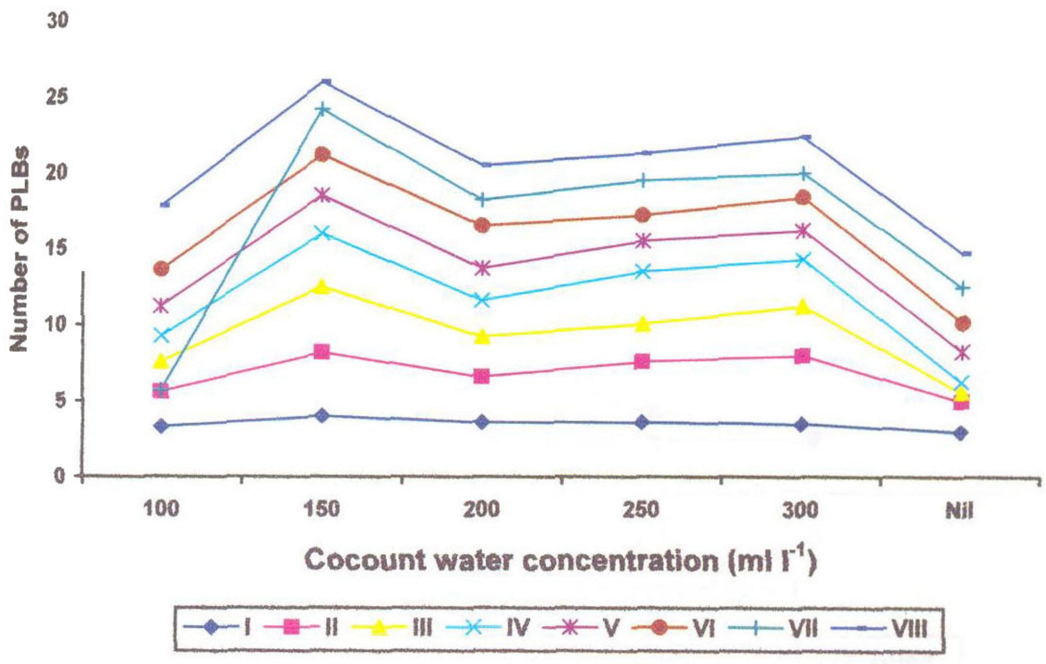


Figure 3: Effect of Coconut water on the *in vitro* proliferation of protocorm like bodies

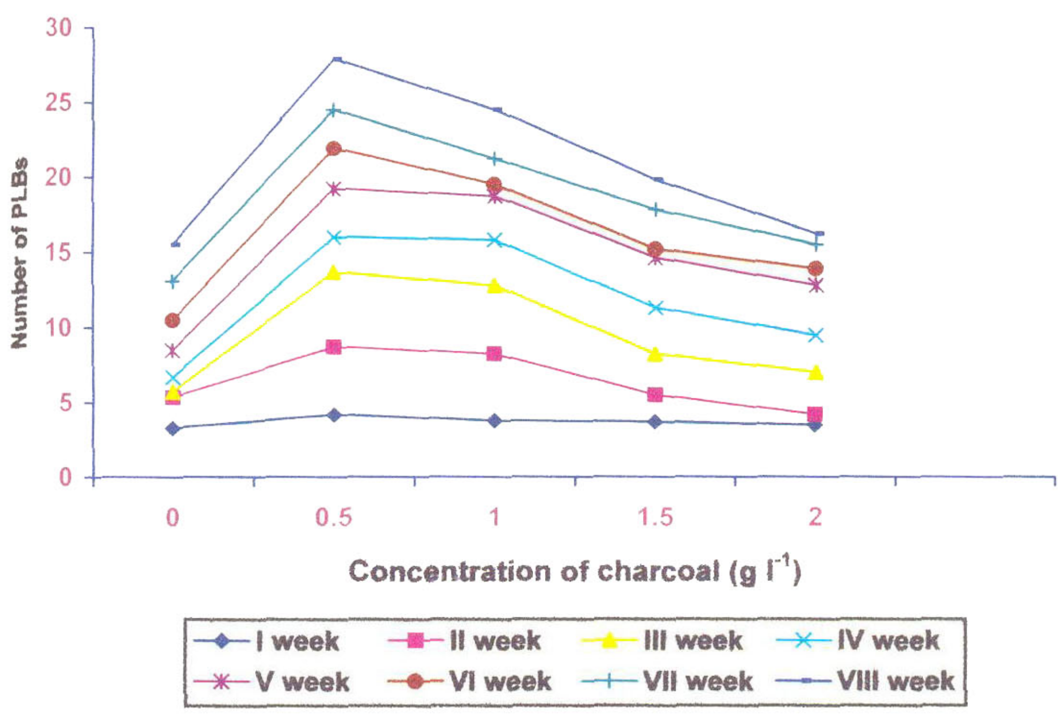


Figure 4: Effect of activated charcoal on the *in vitro* proliferation of protocorm like bodies (plbs)

Table 7 Effect of growth regulators on *in vitro* proliferation of the protocorm like bodies

Medium: MS (½ strength) + sucrose 30gl^{-1} + CW 150 ml l^{-1}+ agar 6gl^{-1} + activated charcoal 0.5g l^{-1}

S. No.	Growth regulators (mg l^{-1})	Percent establish ment	Proliferation rate of PLBs (Weeks)							
			1	2	3	4	5	6	7	8
1.	NAA 1.5 + BA 1.0	88.02	4.20	9.30	13.70	13.90	14.20	14.50	14.90	15.30
2.	BA 1.0	89.12	4.20	9.40	14.20	15.00	16.30	16.50	19.30	20.20
3.	BA 0.2	90.05	5.40	9.80	14.40	18.70	21.50	24.90	26.10	28.30
4.	Nil	87.50	4.20	8.70	13.70	16.30	19.20	21.90	24.70	27.90
	$S.E_m$ C.D (0.05)	2.77 8.53	0.26 0.81	0.20 0.63	0.25 0.77	0.37 1.15	0.52 1.62	0.41 1.26	0.96 2.95	0.84 2.58

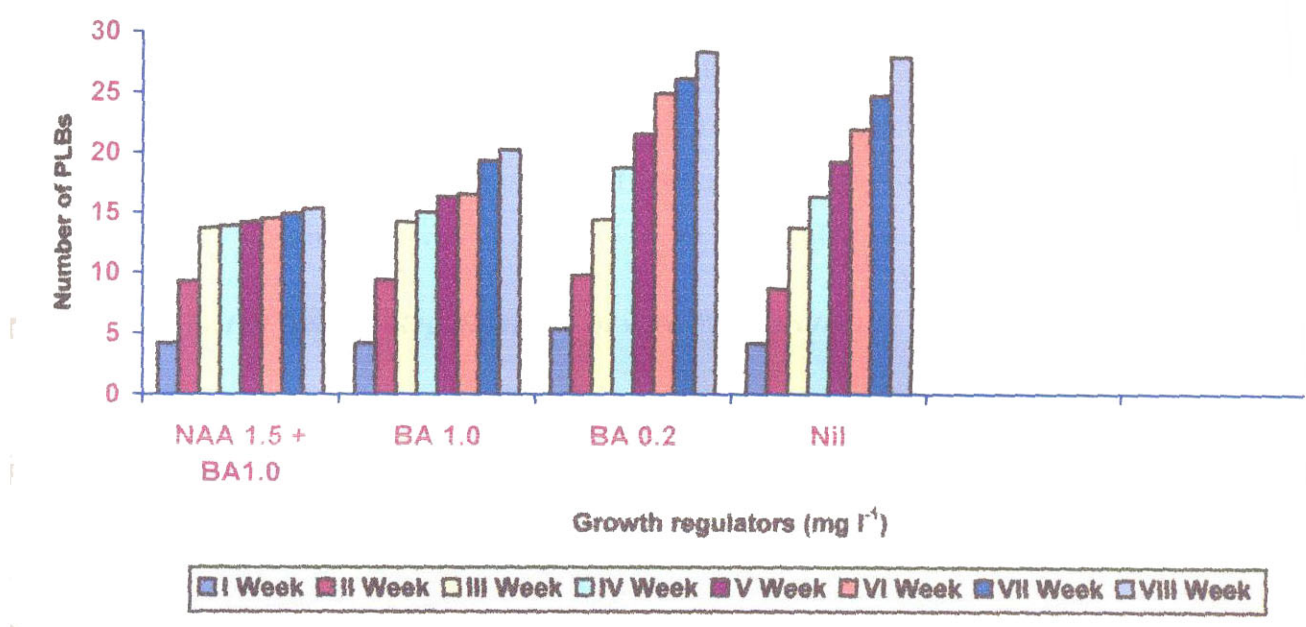


Figure 5: Effect of growth regulators on the *in vitro* proliferation of protocorm like bodies

Out of the various treatments the highest proliferation rate (26.01 in eight weeks) was recorded with coconut water 150 ml l^{-1}. The treatments with coconut water 200 and 250 ml l^{-1} were on par. The control, without the addition of coconut water, recorded the least proliferation rate (14.7).

4.1.2.3. Effect of Activated Charcoal on in vitro Proliferation of PLBs

Significant variation was observed on the establishment percentage and proliferation rate, with the different doses of charcoal (Table 6). Maximum establishment percentage (87.5) was recorded with a concentration of 0.5 g l^{-1}. The highest proliferation rate (27.9 PLBs in eight weeks) was observed with activated charcoal 0.5g l^{-1}. Lesser number of PLBs (15.5) were obtained in the control.

4.1.2.4. Effect of Growth Regulators on in vitro Proliferation of PLBs

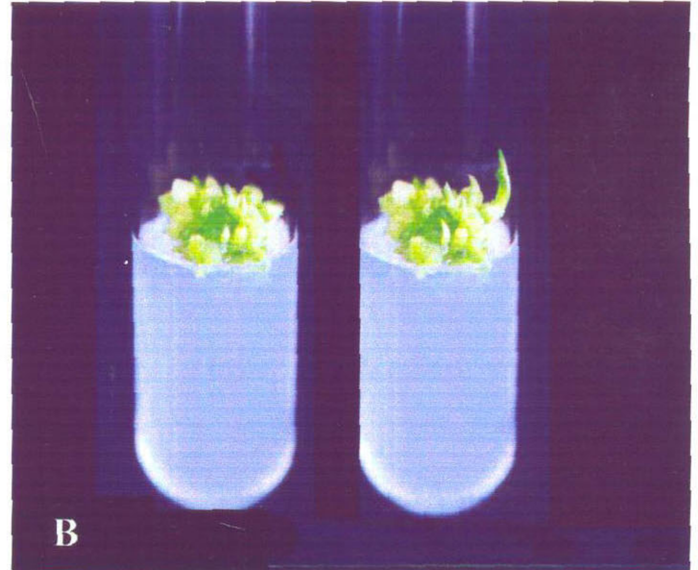
Data recorded on the effect of different growth regulators on the establishment and proliferation rate of PLBs are presented in Table 7. There was significant difference between the various treatments. The maximum culture establishment percentage (90.05) was recorded with BA 0.2 mg l^{-1}. The maximum proliferation rate (28.3) was also observed with BA 0.2 mg l^{-1} and it was on par with the control (27.9). The minimum proliferation rate (15.3) was observed in NAA 1.5 and BA 1.0 mgl^{-1}.

4.2. SENSITIVITY OF ORCHID TISSUES TO ANTIBIOTICS

Experiments were carried out to evaluate the sensitivity of both the protocorms and PLBs to different doses of antibiotics.

4.2.1. Ampicillin

Protocorms were tested for their sensitivity to ampicillin (Table 8). The protocorms were fully green and no change was observed in the first two weeks, in all



A - Protocorms

B - Protocorm like bodies

PLATE 1 : Protocorms and Protocorm like bodies

the treatments. A partial discoloration was observed in the protocorms from the third week onwards and no bleaching was observed upto the sixth week. In the seventh week, bleaching was observed in the protocorms at ampicillin 450 and 500 mg l^{-1}. In the eighth week, the protocorms in ampicillin 400 to 500 mg l^{-1} bleached. After eight weeks protocorms in ampicillin 5 mg l^{-1} recorded the highest survival per cent (58.1) whereas the least (3.5%) was recorded in ampicillin 500 mg l^{-1} . The protocorms in ampicillin 450 to 500 mg l^{-1} did not survive (Table 10).

PLBs were tested for their sensitivity to ampicillin (Table 9). No change was observed in the PLBs during the first four weeks. From the fifth week onwards the PLBs in ampicillin 200 to 500 mg l^{-1} showed partial discoloration. In the eighth week PLBs in ampicillin 400 and 500 mg l^{-1} bleached. Among the PLBs, maximum survival rate (64.5%) was observed in the treatment with ampicillin 5 mg l^{-1}, which was followed by ampicillin 50 mg l^{-1} (60.7) after eight weeks. The minimum survival percentage was recorded in ampicillin 500 mg l^{-1}.

4.2.2. Rifampicin

Sensitivity of *Dendrobium* protocorms to different doses of rifampicin is presented in Table 11. In the first two weeks the protocorms in 5-500 mg l^{-1} rifampicin were green in colour. Partial discoloration was observed in the protocorms from the third week onwards. In the fifth week, bleaching was observed in protocorms with rifampicin 450 to 500 mg l^{-1}. In the seventh week, bleaching was observed in protocorms in 400 mg l^{-1}. Browning was recorded in the protocorms with 450 and 500 mg l^{-1} at the end of eighth week. The maximum survival rate of protocorms (56.9%) was recorded with rifampicin 5 mg l^{-1} (Table 13). The minimum (10.9%) was observed with rifampicin 400 mg l^{-1}. The protocorms in rifampicin 450 and 500 mg l^{-1} did not survive.

Table 8 Sensitivity of *Dendrobium* protocorms to different doses of ampicillin

S. No.	Ampicillin (mgl^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Control	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	+++	+++	+++	+++	+++	+++
3.	50	++++	++++	+++	+++	+++	+++	+++	+++
4.	100	++++	++++	+++	+++	+++	+++	+++	+++
5.	150	++++	++++	+++	+++	+++	+++	+++	+++
6.	200	++++	++++	+++	+++	+++	+++	+++	+++
7.	250	++++	++++	+++	+++	+++	+++	+++	+++
8.	300	++++	++++	+++	+++	+++	+++	+++	+++
9.	350	++++	++++	+++	+++	+++	+++	+++	+++
10.	400	++++	++++	+++	+++	+++	+++	+++	++
11.	450	++++	++++	+++	+++	+++	+++	++	++
12.	500	++++	++++	+++	+++	+++	+++	++	++

Mean of three replications

++++ Fully green

+++ Partial discoloured

++ Bleached

\+ Turning brown and dead

Table 9 Sensitivity of *Dendrobium* PLBs to different doses of ampicillin

S. No.	Ampicillin (mgl^{-1})	Sensitivity (weeks)							
		I Week	II Week	III Week	IV Week	V Week	VI Week	VII Week	VIII Week
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	++++	++++	+++
4.	100	++++	++++	++++	++++	++++	++++	++++	+++
5.	150	++++	++++	++++	++++	++++	++++	++++	+++
6.	200	++++	++++	++++	++++	+++	+++	+++	+++
7.	250	++++	++++	++++	++++	+++	+++	+++	+++
8.	300	++++	++++	++++	++++	+++	+++	+++	+++
9.	350	++++	++++	++++	++++	+++	+++	+++	+++
10.	400	++++	++++	++++	++++	+++	+++	+++	+++
11.	450	++++	++++	++++	++++	+++	+++	+++	++
12.	500	++++	++++	++++	++++	+++	+++	+++	++

Mean of three replications

++++ Fully green

+++ Partial discoloued

++ Bleached

\+ Turning brown and dead

Table 10 Survival of D*endrobium* tissues in ampicillin

S.No	Ampicillin (mg l^{-1})	Survival Per cent (after 8 weeks)	
		PLBs	Protocorms
1.	0	100.0	100.0
2.	5	64.5	58.1
3.	50	60.7	57.5
4.	100	51.4	48.9
5.	150	49.2	44.7
6.	200	41.8	35.2
7.	250	35.2	22.8
8.	300	22.2	20.0
9.	350	12.1	11.5
10.	400	9.8	8.0
11.	450	5.2	0
12.	500	3.5	0

Table 11 Sensitivity of *Dendrobium* protocorms to different doses of rifampicin

S. No.	Rifampicin (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	+++	+++	+++	+++	+++	+++
3.	50	++++	++++	+++	+++	+++	+++	+++	+++
4.	100	++++	++++	+++	+++	+++	+++	+++	+++
5.	150	++++	++++	+++	+++	+++	+++	+++	+++
6.	200	++++	++++	+++	+++	+++	+++	+++	+++
7.	250	++++	++++	+++	+++	+++	+++	+++	+++
8.	300	++++	++++	+++	+++	+++	+++	+++	+++
9.	350	++++	++++	+++	++++	+++	+++	+++	+++
10.	400	++++	++++	+++	+++	+++	++	++	++
11.	450	++++	++++	+++	+++	++	++	++	++
12.	500	++++	++++	+++	+++	++	++	++	++

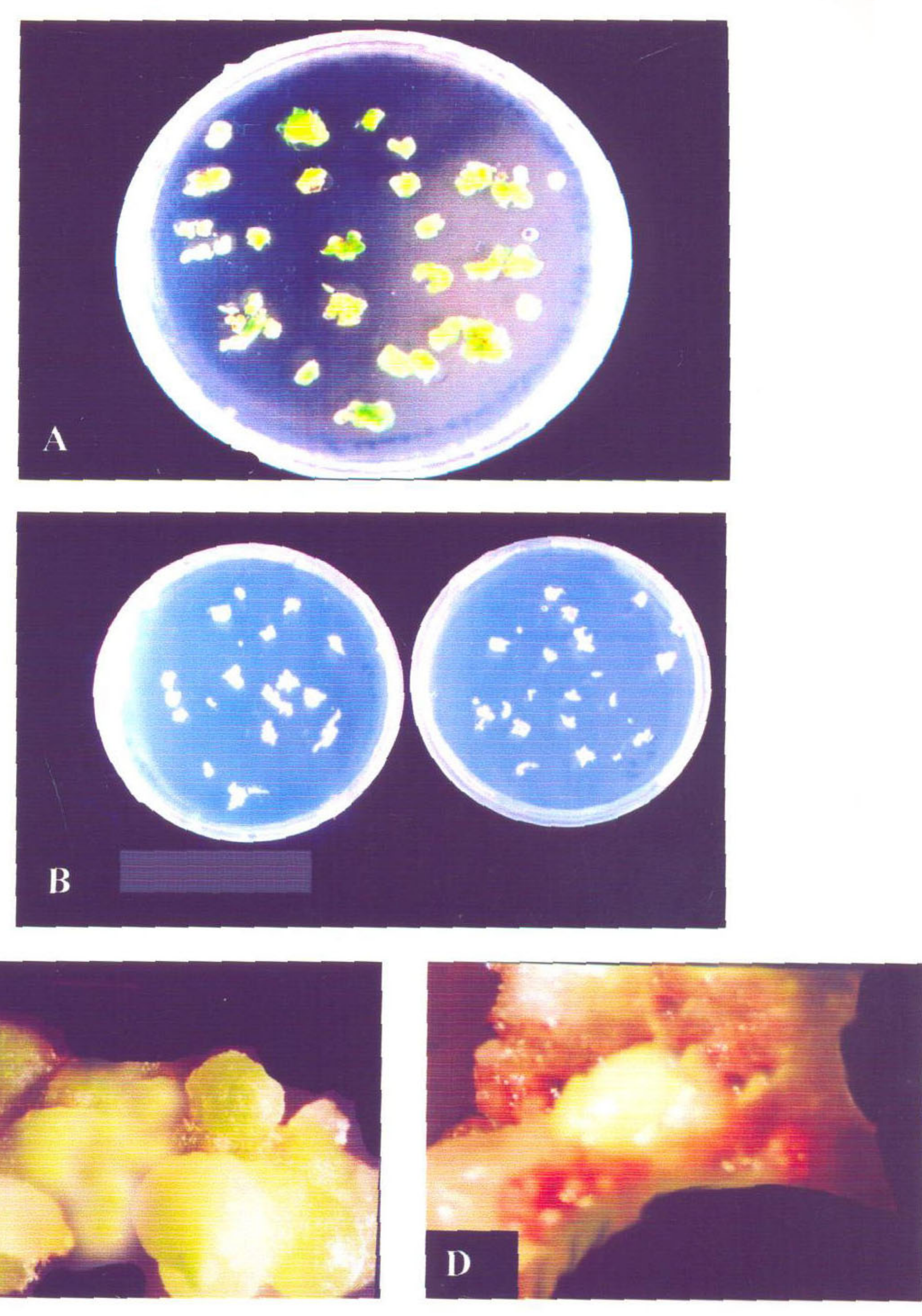
Mean of three replications

++++ Fully green

+++ Partial discoloured

++ Bleached

\+ Turning brown and dead



A - Control

B – Ampicillin 500 mg l^{-1}

C - Ampicillin 5 mg l^{-1}

D – Ampicillin 500 mg l^{-1}

PLATE 2 : Sensitivity of *Dendrobium* tissues to ampicillin

Table 12 Sensitivity of *Dendrobium* PLBs to different doses of rifampicin

S. No.	Rifampicin (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	++++	++++	++++
4.	100	++++	++++	++++	++++	++++	++++	++++	+++
5.	150	++++	++++	++++	++++	+++	+++	+++	+++
6.	200	++++	++++	+++	+++	+++	+++	+++	+++
7.	250	++++	++++	+++	+++	+++	+++	+++	+++
8.	300	++++	++++	+++	+++	+++	+++	+++	+++
9.	350	++++	++++	+++	+++	+++	+++	+++	+++
10.	400	++++	++++	+++	+++	+++	+++	+++	+++
11.	450	++++	++++	+++	+++	+++	+++	+++	++
12.	500	++++	++++	+++	+++	+++	+++	+++	++

Mean of three replications

++++ Fully green

+++ Partial discoloured

++ Bleached

\+ Turning brown and dead

Table 13 Survival of *Dendrobium* tissues in rifampicin

S.No	Rifampicin (mg l^{-1})	Survival Per cent (after 8 weeks)	
		PLBs	Protocorms
1.	0	100.0	100.0
2.	5	63.8	56.9
3.	50	63.0	54.1
4.	100	49.8	49.0
5.	150	47.2	45.2
6.	200	45.6	43.1
7.	250	44.2	40.3
8.	300	35.8	31.7
9.	350	26.7	25.0
10.	400	15.4	10.9
11.	450	10.2	0
12.	500	8.8	0

No change was observed in *Dendrobium* PLBs with rifampicin 5-500 mg l^{-1} during the first two weeks (Table 12). In the third week, the PLBs in 200 - 500 mgl^{-1} rifampicin were partially discolored. Bleaching was observed in the PLBs in rifampicin 450 and 500 mg l^{-1} in the eighth week. The PLBs in rifampicin 5 mg l^{-1} recorded the maximum 63.8 percentage survival (63.8%). The survival per cent of the PLBs was reduced gradually with progressive increase in the concentration of the antibiotic and the highest dose of rifampicin 500 mg l^{-1} showed the minimum survival per cent (8.8%).

4.2.3 Cefotaxime

Sensitivity of *Dendrobium* protocorms to different doses of cefotaxime was studied and the data are presented in Table14. In all the treatments the protocorms were green in colour in the initial two weeks. In the third week, the protocorms in cefotaxime 500 mg l^{-1} showed partial discoloration. Partial discoloration was recorded in protocorms in cefotaxime 400 - 500 mg l^{-1} in the fourth week. From the sixth week, onwards the protocorms in cefotaxime 350 mg l^{-1} bleached. The protocorms in cefotaxime 500 mg l^{-1} turned brown in the seventh week. In the eighth week protocorms in cefotaxime 350-500 mg l^{-1} turned brown and died. It was observed that the protocorms in cefotaxime 5 mg l^{-1} recorded the highest survival rate (66.3%). The protocorms in cefotaxime 300 mg l^{-1} showed the lowest survival (45.8%). Browning was recorded on the sides of protocorms which extended to the centre of the tissues and subsequent death was observed with cefotaxime 350-500 mg l^{-1}. No change was observed in the *Dendrobium* PLBs in cefotaxime 5-500 mg l^{-1} in the initial three weeks (Table15). In the fourth week, the PLBs in cefotaxime 500 mg l^{-1} showed partial discoloration. Partial discoloration and bleaching were observed with the PLBs in cefotaxime 350-450 and, 500 mg l^{-1} respectively in the sixth week. The PLBs in cefotaxime 350-500 mgl^{-1} bleached in the seventh week. Browning of the PLBs was observed with cefotaxime 450 and 500 mg l^{-1} in the eighth week.

Table 14 Sensitivity of *Dendrobium* protocorms to different doses of cefotaxime

S. No.	Cefotaxime (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	++++	++++	++++
4.	100	++++	++++	++++	++++	++++	++++	++++	++++
5.	150	++++	++++	++++	++++	++++	++++	++++	++++
6.	200	++++	++++	++++	++++	++++	++++	++++	++++
7.	250	++++	++++	++++	++++	++++	++++	++++	++++
8.	300	++++	++++	++++	++++	++++	++++	++++	++++
9.	350	++++	++++	++++	++++	+++	++	++	+
10.	400	++++	++++	++++	+++	+++	++	++	+
11.	450	++++	++++	++++	+++	+++	++	++	+
12.	500	++++	++++	+++	+++	+++	++	+	+

Mean of three replications

++++ Fully green
+++ Partial discoloured
++ Bleached
+ Turning brown and dead

Table 15 Sensitivity of *Dendrobium* PLBs to different doses of cefotaxime

S. No.	Cefotaxime (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	++++	++++	++++
4.	100	++++	++++	++++	++++	++++	++++	++++	++++
5.	150	++++	++++	++++	++++	++++	++++	++++	++++
6.	200	++++	++++	++++	++++	++++	++++	++++	++++
7.	250	++++	++++	++++	++++	++++	++++	++++	++++
8.	300	++++	++++	++++	++++	++++	++++	++++	++++
9.	350	++++	++++	++++	++++	++++	+++	++	++
10.	400	++++	++++	++++	++++	++++	+++	++	++
11.	450	++++	++++	++++	++++	+++	+++	++	+
12.	500	++++	++++	++++	+++	+++	++	++	+

Mean of three replications

++++ Fully green

+++ Partial discoloured

++ Bleached

\+ Turning brown and dead

Table 16 Survival of *Dendrobium* tissues in cefotaxime

S.No	Cefotaxime (mg l^{-1})	Survival Per cent (after 8 weeks)	
		PLBs	Protocorms
1.	0	100.0	100.0
2.	5	69.6	66.3
3.	50	68.4	63.8
4.	100	66.4	63.1
5.	150	65.0	60.6
6.	200	60.7	58.2
7.	250	55.3	53.4
8.	300	53.2	45.8
9 .	350	49.60	0
10.	400	42.7	0
11.	450	0	0
12.	500	0	0

The maximum survival rate (69.6%) was recorded with the PLBs in cefotaxime 5 mg l^{-1}. The rate of survival decreased with progressive increase in the concentration of cefotaxime. The minimum survival rate (42.7) was recorded with the PLBs in cefotaxime 400 mg l^{-1}. At high concentration of cefotaxime 450 to 500 mg l^{-1}, none of the PLBs survived. In these PLBs, darkening of the tissues was observed which extended to the centre and death of the PLBs occurred.

4.2.4 Carbenicillin

Protocorms were green in all the treatments for the first two weeks .In the third week the protocorms in carbenicillin 400 mg l^{-1} showed partial discoloration. Partial discoloration was recorded in the protocorms with carbenicillin 350-500 mg l^{-1}in the fourth week. Discoloration and bleaching were observed with carbenicillin 200-300 and 350-500 mg l^{-1} respectively in the sixth week. In the seventh week the protocorms in carbenicillin 250-500 mg l^{-1} got bleached. Browning was recorded in protocorms with carbenicillin 350-500 mg l^{-1} in the eighth week (Table 17)

At the end of the eighth week, 66.9 per cent protocorms survived with carbenicillin 5 mg l^{-1} (Table19). The survival rate decreased with progressive increase in the concentration of carbenicillin and the minimum survival (49%) was recorded with carbenicillin 300 mg l^{-1}. High concentration of carbenicillin from 350-500 mg l^{-1} completely suppressed the growth of the protocorms.

Table 17 Sensitivity of *Dendrobium* protocorms to different doses of carbenicillin

S. No.	Carbenicillin (mg l^{-1})	Scoring for sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	++++	++++	++++
4.	100	++++	++++	++++	++++	++++	++++	++++	++++
5.	150	++++	++++	++++	++++	++++	++++	++++	++++
6.	200	++++	++++	++++	++++	++++	+++	+++	+++
7.	250	++++	++++	++++	++++	+++	+++	++	++
8.	300	++++	++++	++++	++++	+++	+++	++	++
9.	350	++++	++++	++++	+++	+++	++	++	+
10.	400	++++	++++	+++	+++	+++	++	++	+
11.	450	++++	++++	+++	+++	+++	++	++	+
12.	500	++++	++++	+++	+++	+++	++	++	+

Mean of three replications

++++ Fully green
+++ Partial discoloured
++ Bleached
+ Turning brown and dead

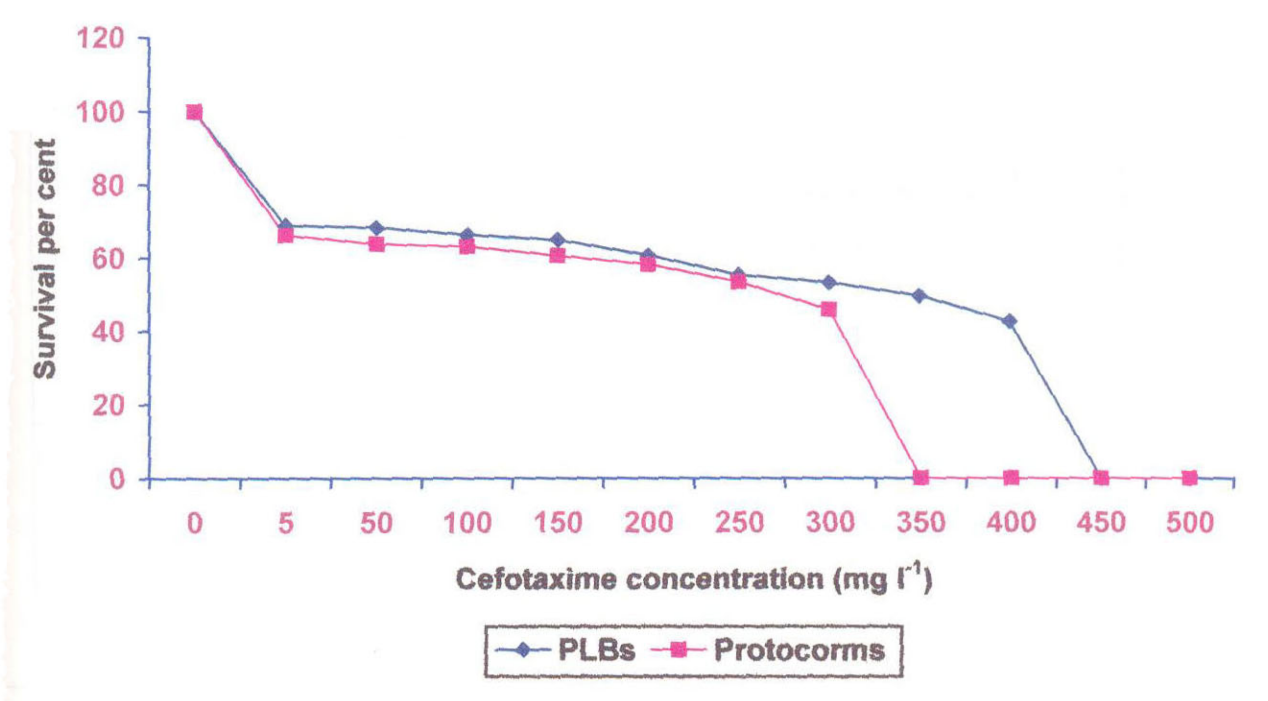


Figure 6. Survival of *Dendrobium* tissues in cefotaxime

Table 18 Sensitivity of *Dendrobium* PLBs to different doses of carbenicillin

S. No.	Carbenicillin (mg l^{-1})	Scoring for sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	++++	++++	++++
4.	100	++++	++++	++++	++++	++++	++++	++++	++++
5.	150	++++	++++	++++	++++	++++	++++	++++	++++
6.	200	++++	++++	++++	++++	++++	++++	++++	++++
7.	250	++++	++++	++++	++++	++++	++++	++++	++++
8.	300	++++	++++	++++	++++	++++	+++	+++	+++
9.	350	++++	++++	++++	++++	++++	+++	+++	++
10.	400	++++	++++	++++	++++	+++	+++	++	+ +
11.	450	++++	++++	++++	+++	+++	+++	++	+
12.	500	++++	++++	++++	+++	+++	+++	++	+

Mean of three replications

++++ Fully green

+++ Partial discolouration

++ Bleached

\+ Turning brown and dead

Table 19 Survival of *Dendrobium* tissues in carbenicillin

S.No	Carbenicillin (mg l^{-1})	Survival Per cent (after 8 weeks)	
		PLBs	Protocorms
1.	0	100.0	100.0
2.	5	68.6	66.9
3.	50	67.2	64.8
4.	100	63.8	62.2
5.	150	61.4	60.1
6.	200	60.4	57.2
7.	250	58.7	53.5
8.	300	55.9	49.0
9.	350	46.5	0
10.	400	43.8	0
11.	450	0	0
12.	500	0	0

No change was recorded in PLBs in all the treatments for the initial three weeks (Table18). In the fourth week, the PLBs in carbenicillin 450 and 500 mg l^{-1} showed a partial discoloration. Partial discoloration was observed in PLBs with carbenicillin 400-500 mg l^{-1} in the fifth week and 300-500 mg l^{-1} in the sixth week. In the seventh week bleaching was observed in the PLBs in carbenicillin 400-500 mgl^{-1}. At the end of eighth week the PLBs in carbenicillin 5-250 mg l^{-1} remained green. The PLBs in 300mgl^{-1} were partially discoloured. The PLBs in 350 and 400 mg l^{-1} got bleached and the PLBs in carbenicillin 450 and 500 mg l^{-1} turned brown.

The PLBs in carbenicillin 5 mg l^{-1} showed the highest survival rate (68.6%). The minimum survival rate (43.8%) was recorded with the PLBs in carbenicillin 400 mg l^{-1}. The PLBs in carbenicillin 450-500 mg l^{-1} showed browning and subsequently death was observed with high concentration of carbenicillin.

4.2.5. Kanamycin

The sensitivity of *Dendrobium* protocorms to different doses of Kanamycin was studied (Table 20) Partial discoloration was observed in the protocorms, with kanamycin 300-500 mg l^{-1} in the first week. In the second week, partial discoloration was observed from kanamycin 150 to 500 mg l^{-1}. Bleaching was observed from the third week onwards in the protocorms with kanamycin 400 mg l^{-1}. In the fourth week bleaching was recorded with the protocorms in kanamycin 350 mg l^{-1}. Bleaching was recorded with kanamycin 300 mg l^{-1} in the fifth week. In the sixth week, the protocorms with 100 mg l^{-1} kanamycin bleached, and those with 400 to 500 mg l^{-1} kanamycin turned brown and died. From the seventh week the protocorms in kanamycin 50 mg l^{-1} bleached and those in kanamycin 350 mg l^{-1} turned brown and died.

Table 20 Sensitivity of *Dendrobium* protocorms to different doses of kanamycin

S. No.	Kanamycin (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	+++	++	++
4.	100	++++	++++	++++	++++	+++	++	++	++
5.	150	++++	+++	+++	+++	+++	++	++	++
6.	200	++++	+++	+++	+++	+++	++	++	++
7.	250	++++	+++	+++	+++	+++	++	++	+
8.	300	+++	+++	+++	+++	++	++	++	+
9.	350	+++	+++	+++	++	++	++	+	+
10.	400	+++	+++	++	++	++	+	+	+
11.	450	+++	+++	++	++	++	+	+	+
12.	500	+++	+++	++	++	++	+	+	+

Mean of three replications

++++ Fully green
+++ Partial discoloured
++ Bleached
+ Turning brown and dead

Table 21 Sensitivity of *Dendrobium* PLBs to different doses of kanamycin

S. No.	Kanamycin (mg l^{-1})	Sensitivity (Weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	++++	++++	++++	++++	++++	++++	++++	++++
3.	50	++++	++++	++++	++++	++++	+++	++	++
4.	100	++++	++++	++++	++++	+++	++	++	++
5.	150	++++	++++	+++	+++	+++	++	++	++
6.	200	++++	+++	++++	+++	++	++	++	++
7.	250	++++	+++	+++	+++	++	++	++	++
8.	300	++++	+++	+++	+++	++	++	++	+
9.	350	++++	+++	+++	+++	++	++	++	+
10.	400	+++	+++	+++	++	++	++	++	+
11.	450	+++	+++	+++	++	++	++	+	+
12.	500	+++	+++	+++	++	++	++	+	+

Mean of three replications

++++ Fully green

+++ Partial discoloured

++ Bleached

+ Turning brown and dead

Table 22. Survival of *Dendrobium* tissues in kanamycin

S.No	Kanamycin (mg l^{-1})	Survival per cent (after 8 weeks)	
		PLBs	Protocorms
0	0	100.0	100.0
1.	5	65.6	62.7
2.	50	50.8	43.9
3.	100	45.7	30.5
4.	150	32.9	23.7
5.	200	22.0	14.4
6.	250	15.1	0
7.	300 – 500	0	0

It was observed at the end of eighth week that the PLBs in kanamycin 5 mg l^{-1} recorded the highest survival rate (65.6%). The lowest survival rate (15.1) was recorded with the PLBs in kanamycin 250 mg l^{-1} (Table 4.2.1.5). Increased concentration of kanamycin from 300 to 500 mg l^{-1} suppressed the growth of PLBs.

No change was recorded in the PLBs with kanamycin 50 to 350 mg l^{-1} in the first week (Table 21). The PLBs with 400 to 500 mg l^{-1} kanamycin showed partial discoloration. Partial discoloration was observed in the PLBs with kanamycin 200 – 500 mg l^{-1} in second week, and from 150-500 mg l^{-1} in the third week. Bleaching was observed during fourth week in the PLBs with kanamycin 400 to 500 mg l^{-1}. In the fifth week, bleaching was recorded in the PLBs with kanamycin 200 mg l^{-1}. The PLBs with kanamycin 100-500 mg l^{-1} bleached in the sixth week. From the seventh week, bleaching was observed in the PLBs with kanamycin 50 mg l^{-1}. Browning and death of the tissues was recorded with kanamycin 300 to 500 mg l^{-1} in the eighth week. In the eighth week, a maximum of 62.7 per cent protocorms survived at the concentration of kanamycin, 5 mg l^{-1}. The survival rate decreased with the progressive increase in the concentration of the antibiotic and a minimum rate of survival (14.4) was observed with kanamycin 200 mg l^{-1}. None of the protocorms survived at a higher concentration of kanamycin 250 - 500 mg l^{-1}.

4.2.6. Hygromycin

Sensitivity of *Dendrobium* protocorms to varying concentrations of hygromycin was studied (Table 23). The protocorms in hygromycin 5 to 150 mg l^{-1} were partial discolored. Bleaching was observed in the protocorms with hygromycin 200 to 500 mg l^{-1} during the first week. From the third week, bleaching was observed with hygromycin 50 mg l^{-1}. Browning was recorded in the fifth week in protocorms with hygromycin 400 to 500 mg l^{-1} and in 300 to 500 mg l^{-1} in the sixth week. By the eight week browning and death of protocorms was observed with hygromycin 150 mg l^{-1}.

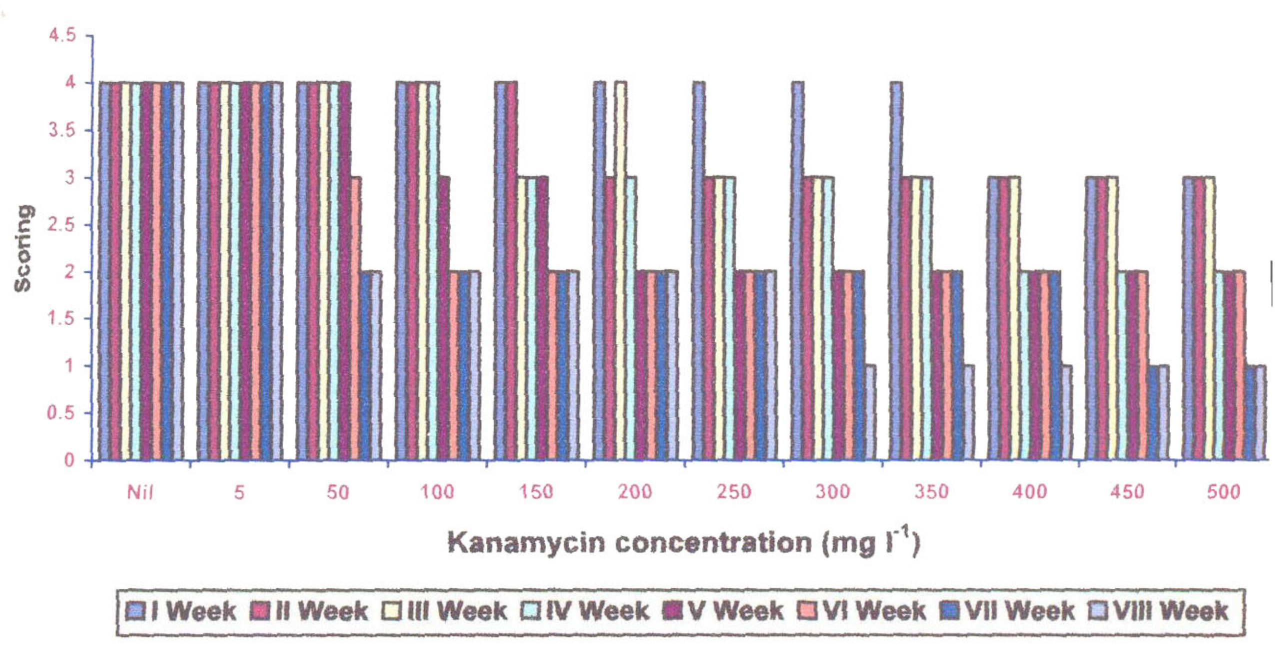
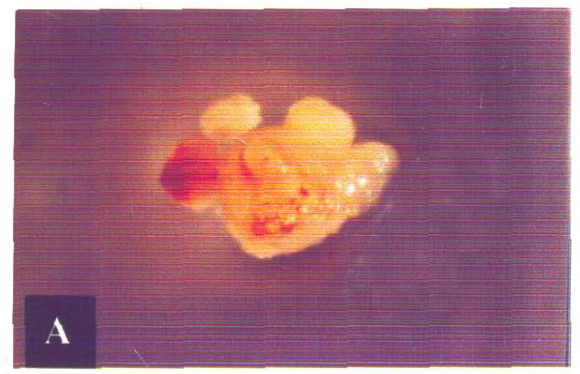
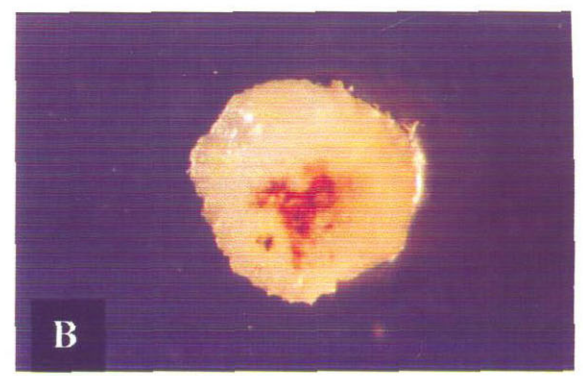


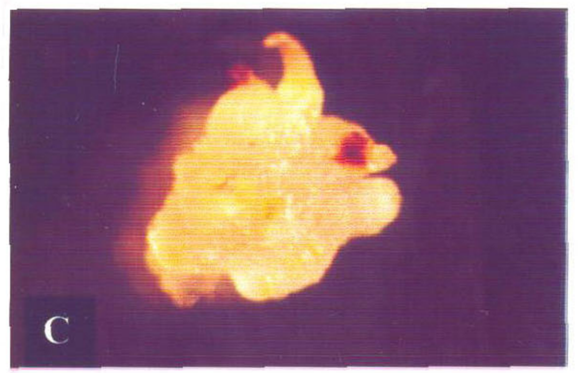
Figure7. Sensitivity of *Dendrobium* PLBs to different doses of kanamycin

Table 23 Sensitivity of *Dendrobium* protocorms to different doses of hygromycin

S. No.	Hygromycin (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	0	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	+++	+++	+++	+++	+++	+++	+++	++
3.	50	+++	+++	++	++	++	++	++	++
4.	100	+++	+++	++	++	++	++	++	++
5.	150	+++	+++	++	++	++	++	++	+
6.	200	++	++	++	++	++	++	+	+
7.	250	++	++	++	++	++	++	+	+
8.	300	++	++	++	++	++	+	+	+
9.	350	++	++	++	++	++	+	+	+
10.	400	++	++	++	++	+	+	+	+
11.	450	++	++	++	++	+	+	+	+
12.	500	++	++	++	++	+	+	+	+





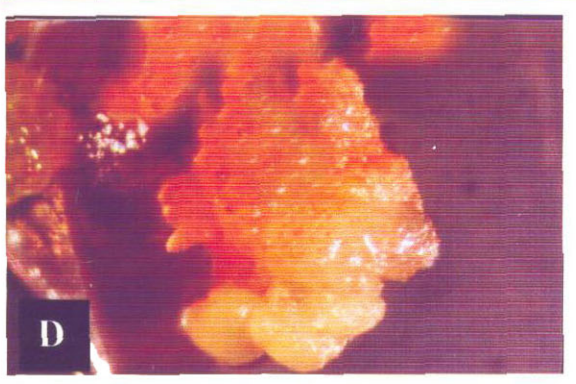


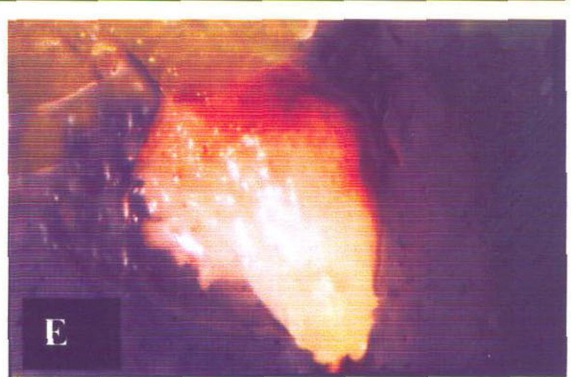
Sensitivity of *Dendrobium* tissues to Rifampicin

A - Rifampicin 5 mg l-1

B - Rifampicin 250 mg l-1

C - Rifampicin 500 mg l-1





D – Kanamycin 50 mg l^{-1}

E – Kanamycin 250 mg l^{-1}

F – Kanamycin 500 mg l^{-1}

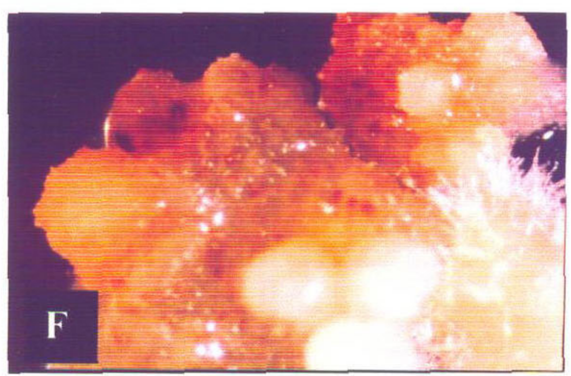


PLATE 3

Sensitivity of *Dendrobium* tissues to kanamycin

Table 24 Sensitivity of *Dendrobium* protocorms to different doses of hygromycin

S. No.	Hygromycin (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	10	+++	+++	+++	+++	+++	+++	+++	+++
3.	20	+++	+++	+++	+++	+++	+++	+++	+++
4.	30	+++	+++	+++	+++	+++	+++	++	++
5.	40	+++	+++	+++	++	++	++	++	++
6.	50	+++	+++	++	++	++	++	++	++
7.	60	+++	+++	++	++	++	++	++	++
8.	70	+++	+++	++	++	++	++	++	++
9.	80	+++	+++	++	++	++	++	++	++
10.	90	+++	+++	++	++	++	++	++	++
11.	100	+++	+++	++	++	++	++	++	++

Table 25 Survival *Dendrobium* tissues in hygromycin

Sl.No	Hygromycin (mg l^{-1})	Final survival Per cent (after 8 weeks)	
		PLBs	Protocorms
1.	5	57.4	51.6
2.	50	26.4	20.2
3.	100	20.0	10.3
4.	150 – 500	0	0

A further study was conducted in protocorms with varying concentrations of hygromycin from 10-100 mg l^{-1} (Table 24). In the initial two weeks the protocorms in all the treatments were partially discoloured. During the third week, the protocorms in hygromycin 50 mg l^{-1} showed bleaching. Bleaching was observed with protocorms in hygromycin 40 mg l^{-1} from the fourth week onwards.

The protocorms in hygromycin 5 mg l^{-1} recorded the maximum survival rate (51.6%). There was a progressive decrease in the per cent of protocorms showing survival when the concentration of hygromycin was increased (Table 25). With hygromycin at 150-500 mg l^{-1} there was no fresh growth and the PLBs turned brown at the sides from the fifth week onwards and subsequently the darkening extended towards the centre and finally the protocorms were dead.

PLBs showed partial discoloration in the treatments with hygromycin 5-450 mg l^{-1} in the first week (Table 26). The PLBs in hygromycin 500 mg l^{-1} bleached in the second week. From the third week, the PLBs in hygromycin 200-500 mg l^{-1} bleached. Bleaching was observed in the PLBs with hygromycin 100 mg l^{-1} from the fourth week. During the fifth week, the PLBs in hygromycin 50 to 250 mg l^{-1} bleached and those with hygromycin 300-500 mg l^{-1} turned brown. At the end of eight week PLBs in hygromycin 5 mg l^{-1} were partially discolored. Bleaching was recorded with PLBs in hygromycin 50 and 100 mg l^{-1}. The PLBs in hygromycin 150 to 500 mg l^{-1} turned brown and died.

Further sensitivity studies with lower doses of hygromycin revealed that the PLBs with hygromycin 10 to 100 mg l^{-1} were partial discolored in the initial three weeks. In the fourth week the PLBs in hygromycin 100 mg l^{-1} showed bleaching. Bleaching was observed with the PLBs in hygromycin 50-100 mg l^{-1} in the fifth week. PLBs in hygromycin 40 mg l^{-1} exhibited bleaching in the seventh week and by eight week the PLBs in hygromycin 30 mg l^{-1} showed bleaching.

Table 26 Sensitivity of *Dendrobium* PLBs to different doses of hygromycin

S. No.	Hygromycin (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	5	+++	+++	+++	+++	+++	+++	+++	+++
3.	50	+++	+++	+++	+++	++	++	++	++
4.	100	+++	+++	+++	++	++	++	++	++
5.	150	+++	+++	+++	++	++	++	++	+
6.	200	+++	+++	++	++	++	++	+	+
7.	250	+++	+++	++	++	++	++	+	+
8.	300	+++	+++	++	++	+	+	+	+
9.	350	+++	+++	++	++	+	+	+	+
10.	400	+++	+++	++	++	+	+	+	+
11.	450	+++	+++	++	++	+	+	+	+
12.	500	++	++	++	++	+	+	+	+

Table 27 Sensitivity of *Dendrobium* PLBs to different doses of hygromycin

S. No.	Hygromycin (mg l^{-1})	Sensitivity (weeks)							
		I	II	III	IV	V	VI	VII	VIII
1.	Nil	++++	++++	++++	++++	++++	++++	++++	++++
2.	10	+++	+++	+++	+++	+++	+++	+++	+++
3.	20	+++	+++	+++	+++	+++	+++	+++	+++
4.	30	+++	+++	+++	+++	+++	+++	+++	++
5.	40	+++	+++	+++	+++	+++	+++	++	++
6.	50	+++	+++	+++	+++	++	++	++	++
7.	60	+++	+++	+++	+++	++	++	++	++
8.	70	+++	+++	+++	+++	++	++	++	++
9.	80	+++	+++	+++	+++	++	++	++	++
10.	90	+++	+++	+++	+++	++	++	++	++
11.	100	+++	+++	+++	++	++	++	++	++

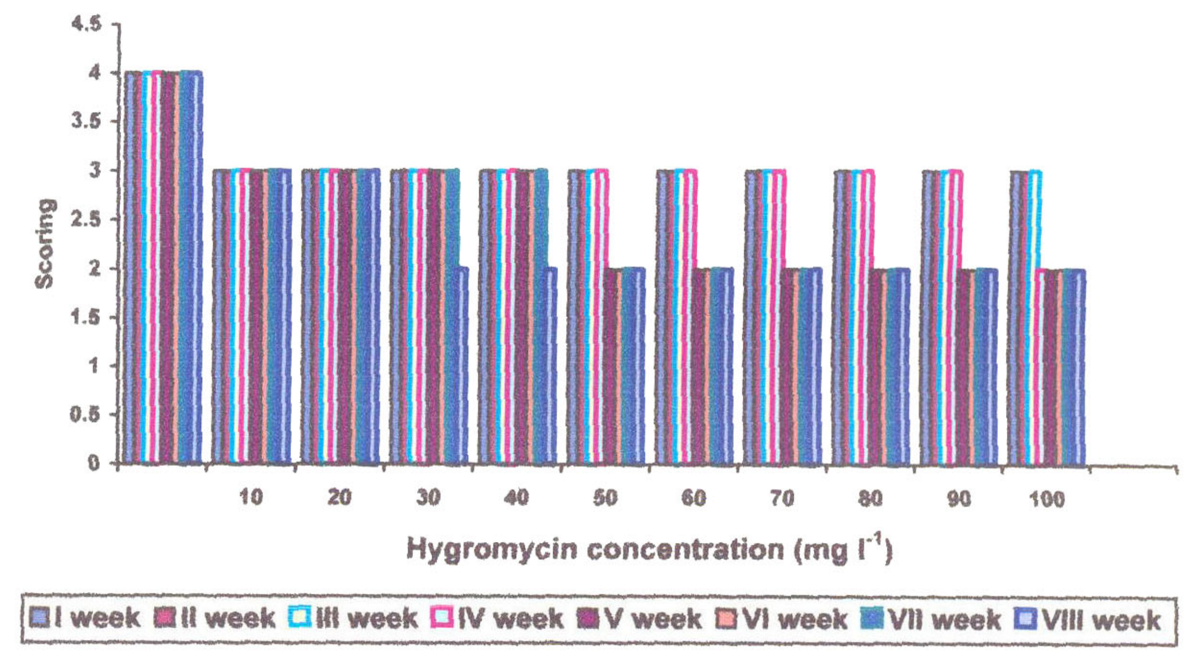


Figure 8 : Sensitivity* of *Dendrobium* protocorms to different doses of hygromycin

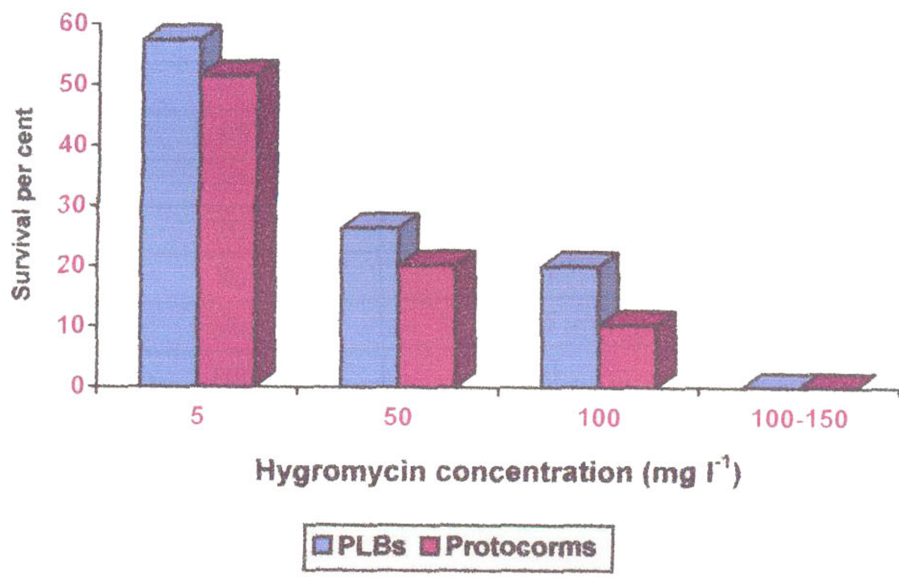


Figure 10. Survival of *Dendrobium* tissues in hygromycin

Table 28 Survival of *Dendrobium* tissues in different doses of hygromycin

S.No	Hygromycin (Mg l^{-1})	Survival Per cent (after 8 weeks)	
		PLBs	Protocorms
	0	100.0	100.0
1.	10	65.5	48.5
2.	20	43.4	37.5
3.	30	39.3	27.0
4.	40	37.4	26.8
5.	50	26.4	20.2
6.	60	23.5	20.0
7.	70	21.0	13.6
8.	80	20.7	13.2
9.	90	20.5	11.0
10.	100	20.0	10.3

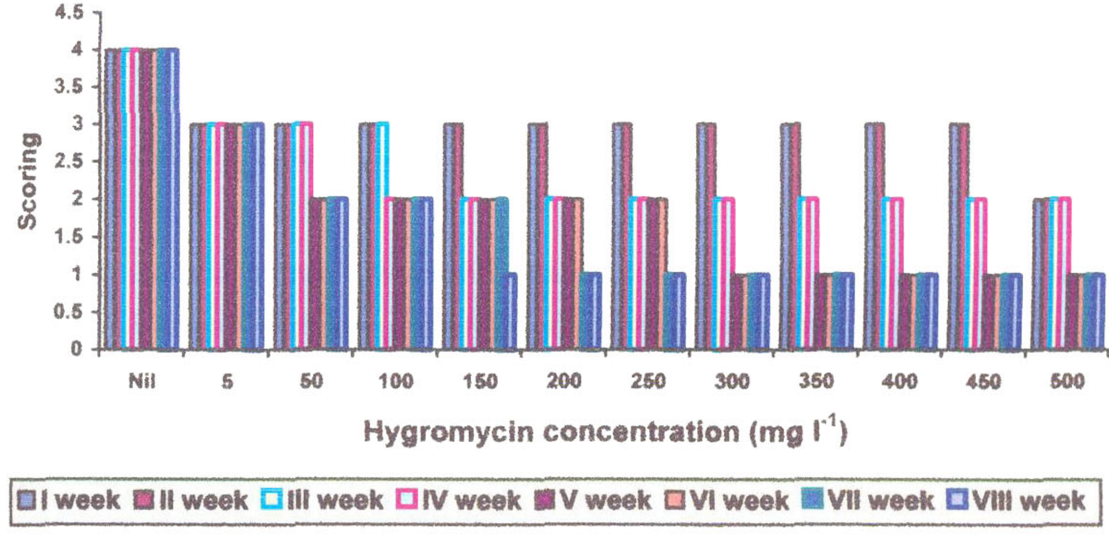


Figure 9 : Sensitivity* of *Dendrobium* PLBs to different doses of hygromycin

PLBs survival to freshly prepared hygromycin at varying concentrations from 5 to 500 mg l^{-1} was tested (Table 27). The minimum survival rate (20.0) was observed at hygromycin 100 mgl^{-1}. Growth was not seen at concentrations of hygromycin 150 mg l^{-1} and above. Thus hygromycin at a concentration of 150 to 500 mg l^{-1} completely suppressed the PLBs growth. The progressive increase in the concentration of hygromycin from 10 to 100 mgl^{-1} decreased the survival rate (Table 28).

4.3. ESTABLISHMENT OF BACTERIAL CULTURE

4.3.1. Growth of *Agrobacterium*

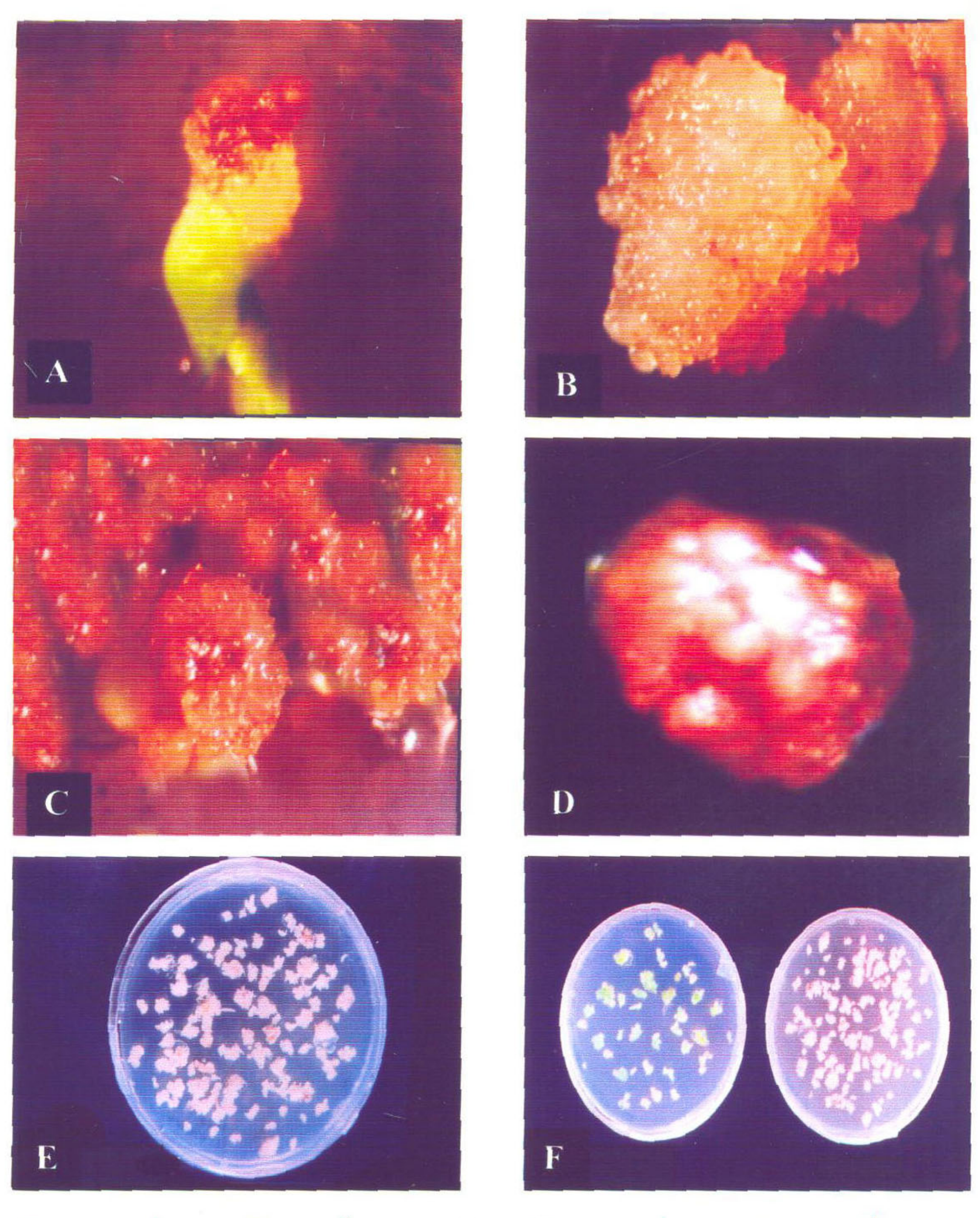
The *Agrobacterium tumefaciens* produced white slimy and smooth colonies. The strain LBA 4404 harbouring PBI 121, pCAMBIA1301 and pCAMBIA 2301 took 38, 35 and 30.8 hours, respectively for growth with colonies. The *A. tumefaciens* strain EHA 105 harbouring the binary plasmids *viz.,* PBI 121, pCAMBIA 1301 and pCAMBIA 2301 showed growth with colonies in 36.5, 30.0 and 29.9 hours respectively (Table 29).

4.4. SCREENING OF *AGROBACTERIUM* STRAINS FOR ANTIBIOTIC SENSITIVITY

The sensitivity of the bacterial strains LBA 4404 and EHA 105 harbouring the plasmids PBI 121, pCAMBIA 1301 and pCAMBIA 2301 to different doses of antibiotics, *viz.,* ampicillin, rifampicin, cefotaxime, carbenicillin, kanamycin and hygromycin was studied. The results of the tests were used in the selection of bacteria with the vectors.

Table 29 Growth of *Agrobacterium* strains

S.No	Bacterial strains	Plasmids	Time taken for growth (Colony formation) (hrs)
1.	LBA 4404	PBI 121	38.0
2.		pCAMBIA 1301	35.0
3.		pCAMBIA 2301	30.8
4.	EHA 105	PBI 121	36.5
5.		pLAMBIA 1301	30.0
6.		pCAMBIA 2301	29.9



A - Hygromycin - 50 mg l^{-1}

B - Hygromycin - 250 mg l^{-1}

C - Hygromycin - 400 mg l^{-1}

D - Hygromycin - 500 mg l^{-1}

E - Control tissues (0 mg l^{-1})

F - Bleached tissues (500 mg l^{-1})

PLATE 4 : Sensitivity of *Dendrobium* tissues to hygromycin

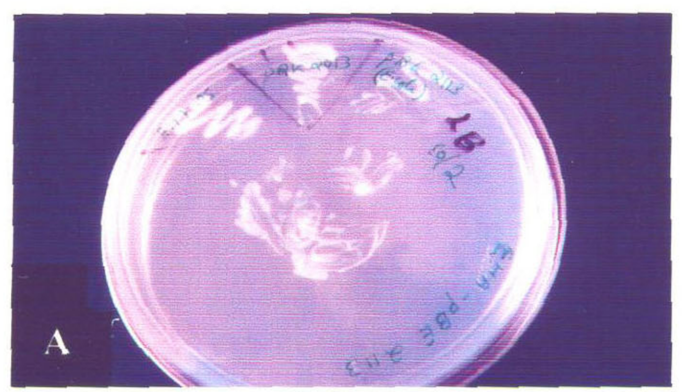
Among the different antibiotics studied PBI 121 was sensitive only to rifampicin and kanamycin. On the third day, the bacterial growth was observed at rifampicin 50 mg l^{-1} and kanamycin50 mg l^{-1}. On the third day the growth of *Agrobacterium* strains, LBA 4404 and EHA 105 harbouring pCAMBIA 1301 was present on plates with rifampicin and hygromycin at 50 mgl^{-1} and kanamycin 100 mgl^{-1}. Bacterial growth was observed in YEP plates with rifampicin 50 mg l^{-1} and kanamycin 100 mg l^{-1} for both the strains, LBA 4404 and EHA 105 harbouring the binary vector pCAMBIA 2301.

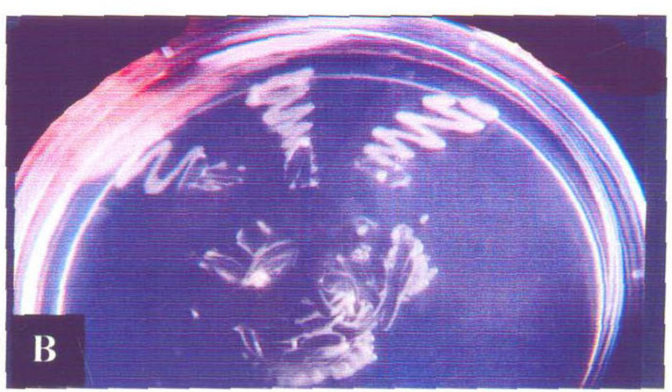
4.4.1. Test for the bacteriocidal activity of cefotaxime

In the present study the bacteriocidal effect of cefotaxime was tested by culturing *A. tumefaciens* in YEP liquid medium containing different doses of cefotaxime ranging from 50 to 500 mg l^{-1}. The bacterial growth was recorded from 50-150 mg l^{-1}. At concentrations 200 mg l^{-1} and above there was absence of bacterial growth. At these concentration the *Dendrobium* explants also did not show any discoloration or bleaching.

4.5. TRIPARENTAL MATING

On the first day of experiment *A. tumefaciens* strain EHA 105 was streaked on AB minimal plate with 20 μg/ml rifampicin. This plate was kept for growth at 28°C for 48 hours. On the second day *E-coli* strain PRK 2013 and PPE 2113 were streaked on LB plates supplemented with kanamycin 50 μg/ml. On the third day triparental mating was performed by mixing EHA 105, PRK 2013 and PPE 2113 in a AB minimal plate without antibiotics. This plate was incubated at 28°C for 48 hours. This mixture was streaked on AB minimal plate with rifampicin 20 μg/ml and kanamycin 50 μg/ml. Simultaneously two control plates were also streaked, one with





A, B – Triparental mating

C – Plate with *Agrobacterium* growth

PLATE 5 : Tri Parental Mating

Table 30 Test for the bacteriocidal activity of cefotaxime

Bacterial growth							
S. No.	Cefotaxime (Mg l^{-1})	PBI 121		pCAMBIA 1301		pCAMBIA 2301	
		EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
1.	0	+	+	+	+	+	+
2.	50	+	+	+	+	+	+
3.	100	+	+	+	+	+	+
4.	150	+	+	+	+	+	+
5.	200	-	-	-	-	-	-
6.	250	-	-	-	-	-	-
7.	300	-	-	-	-	-	-
8.	350	-	-	-	-	-	-
9.	400	-	-	-	-	-	-
10.	450	-	-	-	-	-	-
11.	500	-	-	-	-	-	-

Mean of three replications
+ bacterial growth
- no bacterial growth

AB minimal and rifampicin 20 μg/ml and the other with AB minimal and kanamycin 50 μg/ml. All the plates were inoculated. No growth was observed in two control plates. On the plate with rifampicin 20 μg/ml and kanamycin 50 μg/ml, the transformed *Agrobacterium* survived. A single colony was picked up for further experiments.

4.6. GENETIC TRANSFORMATION IN *DENDROBIUM*

4.6.1. Effect of Size of Explant

Explants of different sizes were co-cultivated to standardize the optimum size for maximum transformation efficiency Table 31.

The use of protocorms as explants did not yield any transformants in all binary plasmids PBI 121, pCAMBIA 1301 and pCAMBIA 2301. The maximum 2.0 percentage are transformants were obtained when the PLBs of 0.2 cm were co-cultivated with EHA 105 harbouring pCAMBIA 2301. The transformation efficiency of 0.5 per cent was obtained when the same plasmid pCAMBIA 2301 was in LBA 4404 and the PLBs of 0.4 and 0.5 cm size were co-cultivated. With LBA 4404 (pCAMBIA 1301), the transformation efficiency of 0.5 per cent was obtained when 0.2 cm PLBs were used for co-cultivation.

4.6.2 Effect of Number of Explants

The optimum number of explants that should be kept in a single petridish (90 mm) during co-cultivation was studied and the results are presented in Table 32. The explants were infected by two methods. One method was the infection with the bacterial pellets obtained after centrifugation of the overnight grown bacterial culture. The second method was infection with 1:5 v/v bacterial suspension and fresh medium.

Table 31 ***Effect of the size of the explants on the transformation efficiency***

Explants (cm)	Percent explants retained												Transformation efficiency					
	After co cultivation						After incubation											
	PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301	
	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
Protocorm																		
0.1	45.2	41.4	62.1	37.2	60.5	68.9	35.7	30.8	37.1	35	52.4	49.7	0	0	0	0	0	0
0.2	43.8	42.8	60.7	35.6	67.3	63.3	35	30.4	34.2	30.8	50.9	48	0	0	0	0	0	0
Control 0.1 – 0.2	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0
PLBs																		
0.1	64.8	63.9	62.5	56.8	69.4	67.4	60.9	59.4	35.8	34.6	51.8	49.9	0	0	0	0	0	0
0.2	62.5	61.8	61.7	55.3	67.5	64.8	61.4	60.2	33.9	31.0	50.0	45.2	0	0	0	0.5	2.0	0.5
0.3	69.3	70.2	59.8	52.9	60.5	57.5	45.3	60.8	35.1	30.3	48.3	38.3	0	0	0	0	0	0
0.4	65.4	60.6	56.2	50.8	58.7	55.4	38.6	60.2	35.0	29.5	45.7	36.9	0	0	0	0	0.5	0
0.5	63.7	61.2	55.0	59.6	55.0	50.7	33.0	49.8	34.4	29.0	41.4	27.8	0	0	0.5	0	0	0
Control 0.1 – 0.5	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0

Co-cultivation time : 3 days

Induction time : 15 minutes

Bacterial agent : Cefotaxime 200 mg l^{-1}

Antibiotics : PBI 121, pCAMBIA 2301-Kanamycin 200 mg l^{-1}

pCAMBIA 1301- Hygromycin 100mg l^{-1}

Table 32 Effect of number of explants per petridish on the transformation efficiency

Explants / petridish		Percent explants retained												Transformation efficiency					
		After co cultivation						After incubation											
		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301	
		EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
20	S	91.4	97.4	90.8	98.2	98.1	94.3	75.3	77.0	81.5	70.0	60.8	50.2	0	0	0	0	0	0
	P	70.3	72.3	72.4	75.0	73.8	72.1	50.9	51.4	51.5	65.8	45.5	40.3	0	0	0	0	0	0
	C	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0
30	S	87.2	86.3	89.4	84.6	88.8	80.4	72.1	70.4	78.4	65.1	58.8	49.7	0	0	0	0.5	0	0
	P	72.4	77.8	70.3	75.9	74.4	73.5	48.3	49.2	49.7	50.5	44.0	39.8	0	0	0	1.0	0	0
	C	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0
40	S	94.5	98.1	95.1	96.9	95.8	94.2	605	58.3	72.9	48.4	55.6	45.4	0	0	0	0	0.5	0
	P	82.3	84.6	82.6	81.2	80.1	83.9	44.6	45.5	45.00	40.3	42.8	37.5	0	0	0	0	1.0	0
	C	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0
50	S	88.4	89.1	87.8	88.7	85.6	87.6	58.7	55.1	70.6	41.5	50.4	42.1	0	0	0	0	0	0
	P	79.5	80.3	81.6	82.5	81.8	81.4	40.1	39.8	43.3	40.4	40.1	35.0	0	0	0	0	0	0
	C	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0

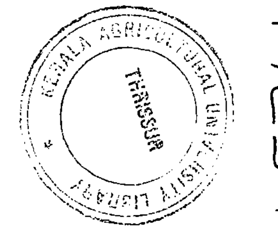
Co-cultivation time : 3 days

Infection time : 15 minutes

Bacterial agent : Cefotaxime 200 mgl^{-1}

Selection agent : PBI 121, pCAMBIA 2301 – Kanamycin 200 mgl^{-1}

pCAMBIA 1301 – Hygromycin 100 mgl^{-1}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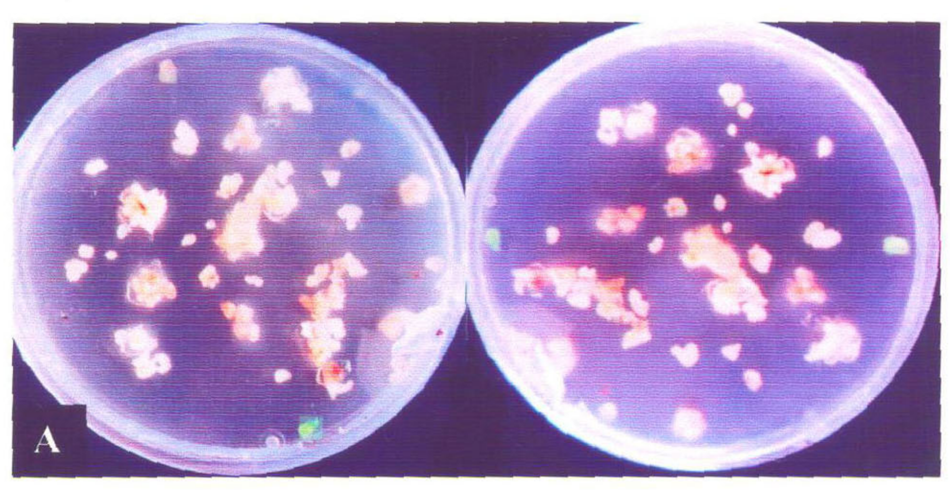
Explants retained after co-cultivation (98.2) was maximum when the explants were infected by using bacterial suspension. But the transformation efficiency was the highest (1.0) in the pelleting method. The explants infected with the bacterial suspension recorded only 0.5 percentage success. In the experiment carried out with 20 explants the transformants (0.5%) were obtained only with the infection of the explants with pellets whereas no transformant was obtained when bacterial suspension was used for infection.

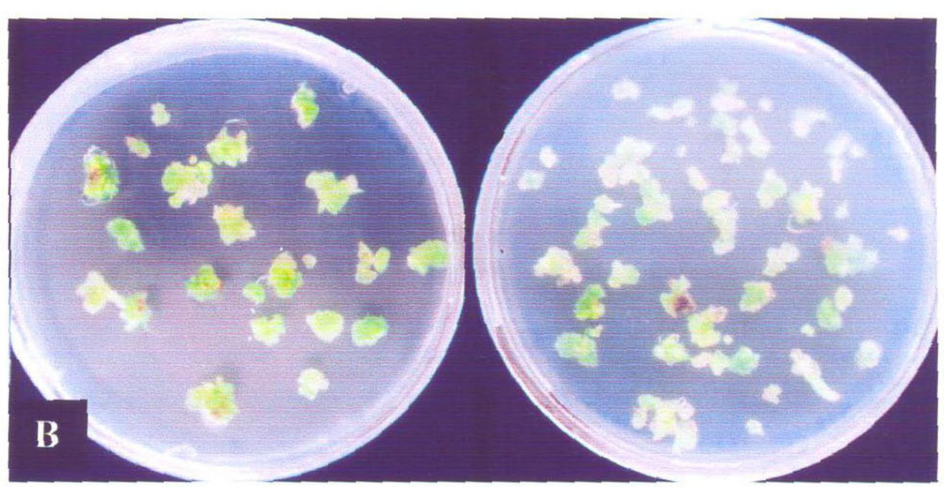
When 30 and 40 explants were kept per (90mm) petridish the transformation efficiency (0.5 to 1 per cent) was the same in both. But when 50 explants were kept in a (90 mm) petriplate over-crowding occurred and the infection spread to the nearby explants and thus overgrowth of bacteria was recorded. This overgrowth was dense and it was not possible to eradicate the overgrown bacteria with the addition of cefotaxime. The orchid tissues were also vitrified.

4.6.3 Effect of Infection Time

The infection was carried out for 10, 15 and 20 minutes so as to standardize the optimum time required for infection (Table 33). Among the different treatments transformants were reported only with 15 minutes infection period. Transformants were obtained with LBA 4404 (PCAMBIA 1301) and EHA 105 (PCAMBIA 2301). In the 15 minutes infection period, transformants of LBA 4404 (PCAMBIA 2301) were obtained only with the infection process by using bacterial pellets. The infection by diluting the bacterial suspension did not produce any transformants.

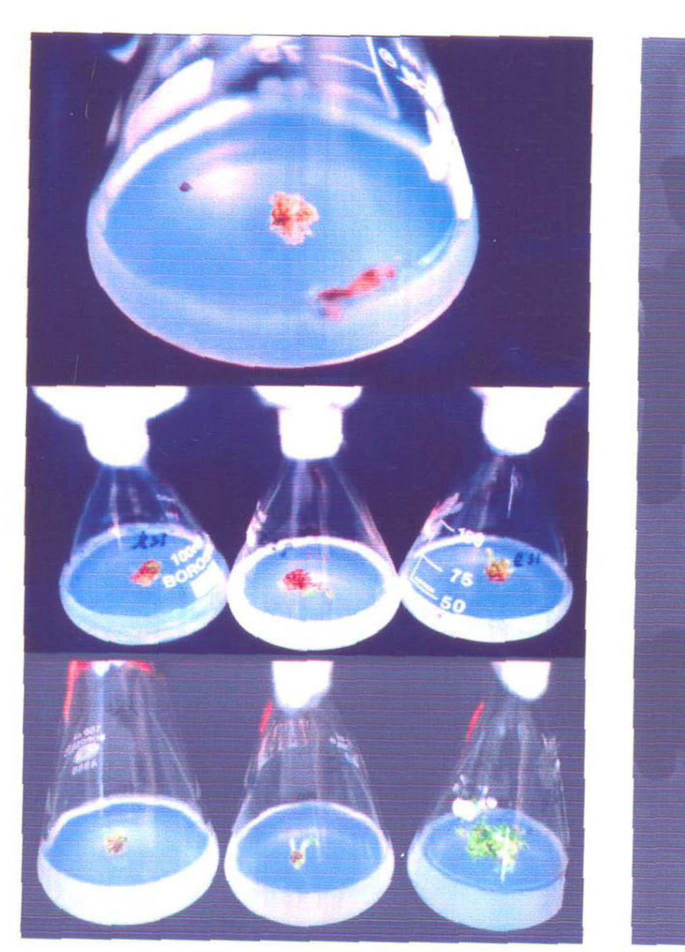
There was variation in the transformation efficiency with the two methods of infection process at the same infection period. In the experiment with 15 minutes infection time, the maximum efficiency (1.0) was with pelleting method. In the same 15 minutes infection with LBA 4404 (PCAMBIA 1301) the transformation efficiency





A & B - Transformed Tissues (green)

PLATE 6 : Tissues in Selection Medium



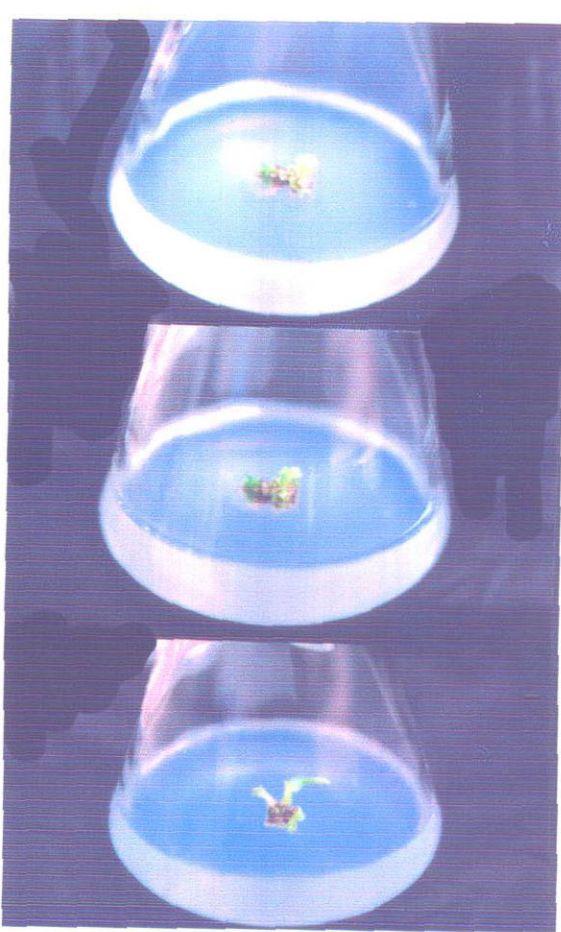


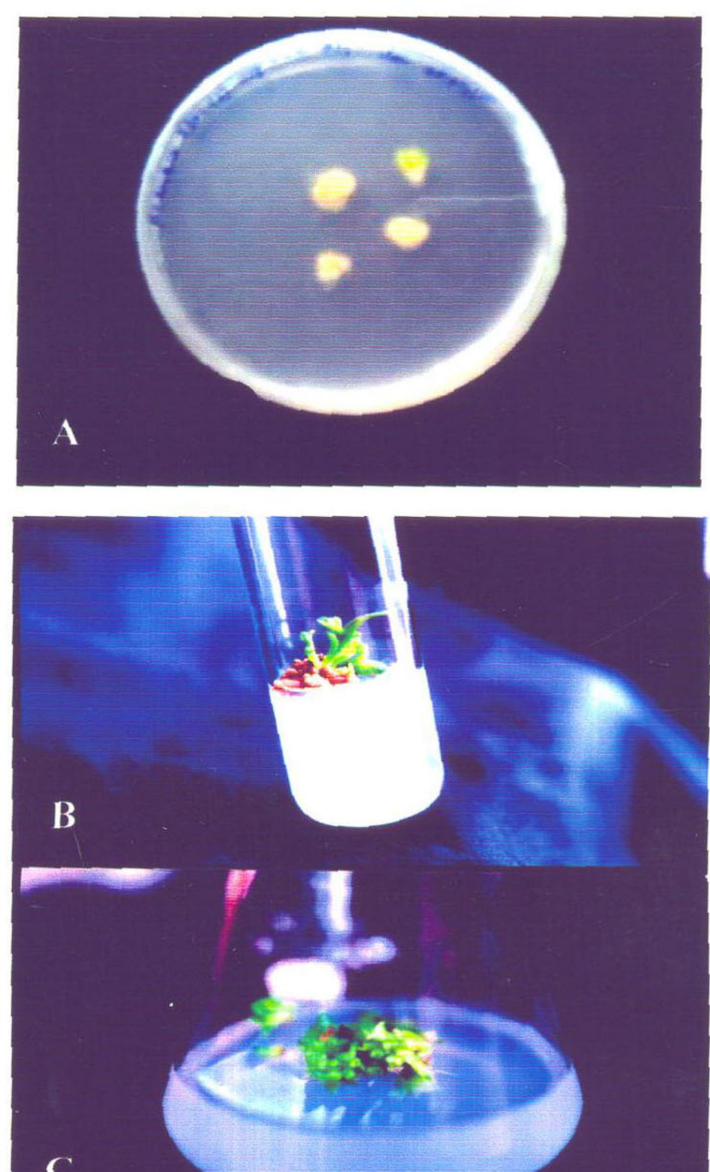
PLATE 7 : Transgenic tissues in different growth stages

Table 33 Effect of infection time on transformation efficiency

Infection time		Percent explants retained												Transformation efficiency					
		After co cultivation						After incubation											
		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301	
		EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
10	S	75.4	78.1	73.4	80.6	70.4	85.2	55.4	56.4	48.6	51.2	50.6	55.7	0	0	0	0	0	0
	P	60.1	63.2	61.5	62.1	60.89	63.1	42.7	47.9	45.5	47.8	42.4	48.8	0	0	0	0	0	0
	C_1	100.0	100	100	100.0	100	100	100	100	100	100.0	100	100	0	0	0	0	0	0
	C_2	100.0	100	100	100.0	100	100	100	100	100	100.0	100	100	0	0	0	0	0	0
15	S	72.0	74.6	73.8	77.8	72.3	78.4	65.4	69.4	62.4	67.9	66.6	67.3	0	0	0	0.5	0.5	0
	P	58.8	60.3	61.1	65.5	62.4	66.5	48.5	49.0	50.8	54.3	52.5	54.5	0	0	0	1.0	1.5	1.0
	C_1	100	100	100	100.0	100	100	100	100	100	100	100	100	0	0	0	0	0	0
	C_2	100	100	100	100.0	100	100	100	100	100	100	100	100	0	0	0	0	0	0
20	S	30.2	32.8	32.6	34.3	32.2	32.4	21.8	24.8	21.7	24.2	18.7	23.5	0	0	0	0	0	0
	P	20.4	24.6	24.4	25.0	22.0	23.2	11.3	12.3	14.3	18.6	13.5	14.2	0	0	0	0	0	0
	C_1	100	100	100	100.0	100	100	100	100	100	100	100	100	0	0	0	0	0	0
	C_2	100	100	100	100.0	100	100	100	100	100	100	100	100	0	0	0	0	0	0

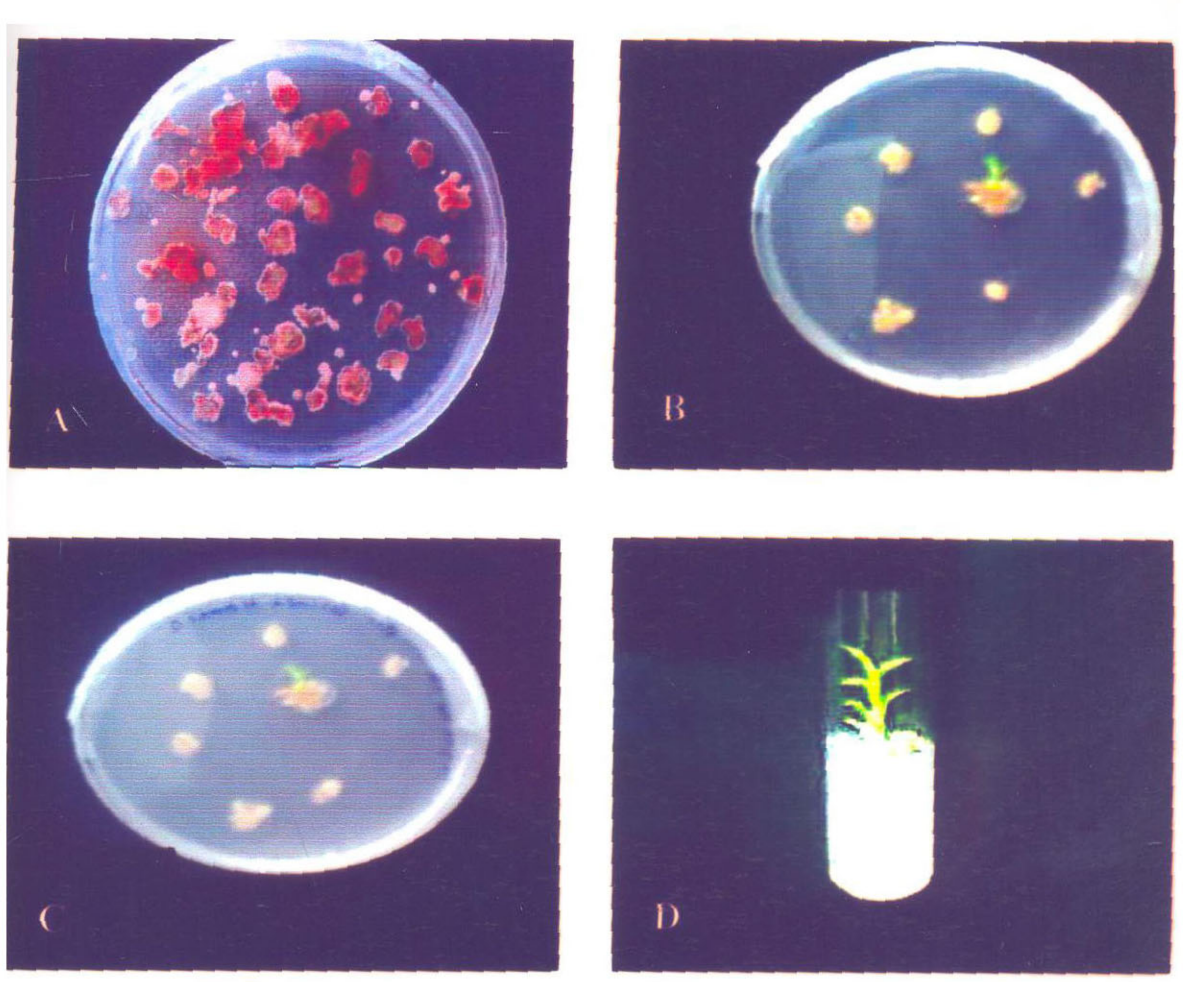
Co-cultivation time : 3 days
Bacterial agent : Cetofaxime 200 mgl^{-1}
Selection agent : PBI 121, pCAMBIA 2301 – Kanamycin 200 mgl^{-1}
pCAMBIA 1301 – Hygromycin 100 mgl^{-1}

C_1 - with antibiotics
C_2 - without antibiotics



A, B, C - Transgenic Plants

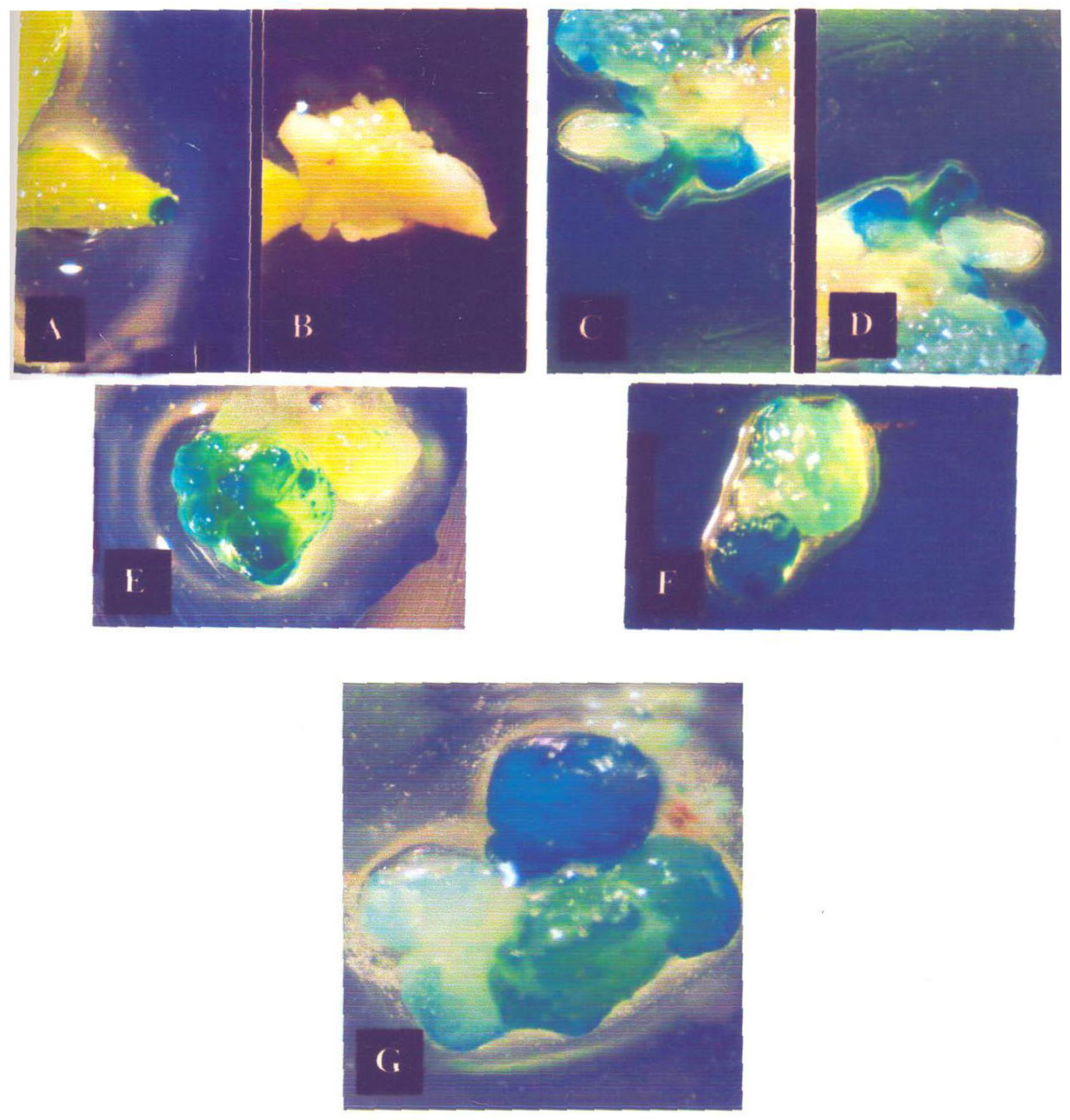
PLATE 8 : Transgenic Plants



A - Tissues with bacterial overgrowth

B, C, D - Transgenic plant

PLATE 9 : Transgenic Plants



A, C to G - Gus Stained Transformed Tissues

B - Untransformed Tissues (Control)

PLATE 10 : Gus Staining

with suspension was 0.5 percentage while the same vector produced 1.0 per cent transformants by using pellets.

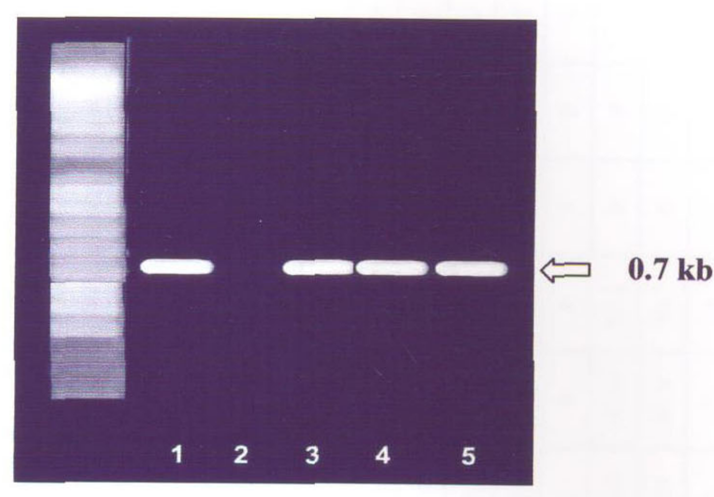
The per cent PLBs resistant to kanamycin (PBI 121, PCAMBIA 2301) and hygromycin (PCAMBIA 1301) were high (18.9) when wounded PLBs were used for infection with bacterial suspension 1:5 v/v. Even when the infection was done with the bacterial pellets the wounded PLBs showed the best response with the recovery of maximum number of resistant PLBs (20.1) when compared to the unwounded (5.3).

4.6.4 Effect of Co-cultivation Days

Agrobacterium with the binary vectors and the plant tissues were co-cultivated in dark for two, three, four, five and six days at 26°C in a culture room. The effect of number of days of co-cultivation on the maximum transformation efficiency was standardized (Table 34).

Among the different treatments, transformants were obtained from the explants co-cultivated with *Agrobacterium* in the dark for 2.0 and 3.0 days. No transformant was produced from the explants co-cultivated for more than three days. Heavy over growth of the bacteria was seen surrounding the explants co-cultivated for more than three days, and when the pertriplates were opened it produced an off smell and the plant tissues were turgid and fragile.

The maximum transformation efficiency (2.0%) was obtained when LBA 4404 (PCAMBIA 1301) and EHA 105 (PCAMBIA 2301) were co-cultivated for three days and the infection was carried out with bacterial pellets obtained by centrifugation of the overnight grown bacterial culture.



1, 3, 4, 5 - Transformed Plants

2 - Untransformed Plant

PLATE 11 : PCR Analysis

Table 34 Effect of co-cultivation on transformation efficiency

No. of days of co-cultivation		Percent explants retained												Transformation efficiency					
		After co cultivation						After incubation											
		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301	
		EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
2	S	17.5	21.2	27.8	21.4	24.8	23.4	19.8	20.1	37.9	25.9	44.9	40.2	0	0	0	0	1.0	0
	P	23.2	24.5	34.6	27.8	30.4	29.5	32.5	25.4	39.5	29.0	50.8	35.8	0	0	0	0	1.5	0.5
	C_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	S	30.0	31.4	36.7	32.4	35.6	33.8	38.0	372	40.2	34.8	60.6	44.5	0	0	0.5	0.5	1.0	0.5
	P	35.7	37.8	47.5	38.0	40.4	39.2	42.5	41.8	49.8	40.2	60.2	50.6	0	0	1.5	2.0	2.0	1.0
	C_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	S	42.50	44.7	49.3	45.4	49.8	47.5	50.5	50.0	52.4	49.6	70.2	60.8	0	0	0	0	0	0
	P	47.8	49.2	53.8	49.8	55.0	45.6	57.9	53.8	55.0	52.1	82.2	75.0	0	0	0	0	0	0
	C_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	S	55.0	56.3	64.2	60.1	69.2	62.3	62.6	61.2	66.8	63.8	75.0	72.8	0	0	0	0	0	0
	P	63.2	65.1	70.9	67.8	74.6	68.0	70.2	68.5	75.4	70.4	84.4	82.3	0	0	0	0	0	0
	C_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	S	60.5	66.2	69.3	67.4	72.9	69.5	70.2	68.1	72.3	75.0	83.0	85.2	0	0	0	0	0	0
	P	75.8	76.5	75.4	78.2	79.0	79.5	80.1	76.1	77.8	80.4	85.0	89.7	0	0	0	0	0	0
	C_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

S - bacterial suspension method (1:5 v/v)
P - pelleting method
C_1 - control with antibiotics
C_2 - control without antibiotic

Transformation efficiency of 1.0 per cent was obtained when the PLBs were co-cultivated with EHA 105 harbouring PCAMBIA 2301, when the bacterial suspension method was used. The same strain with the same vector gave a transformation efficiency of 1.5 percentage when the pelleting method was used for infection.

4.6.5 Effect of Incubation Period

The maximum transformation efficiency of 1.5 percentage was obtained when the tissues were incubated for four days. No transformant was recovered from the tissues incubated for two days. For the tissues incubated for eight and ten days the transformants efficiency of 0.5 percentage was obtained (Table 35).

Table 35 Effect of incubation days on transformation efficiency

No. of days		Percent explants retained						Transformation efficiency (percent)					
		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pLAMBIA 2301	
		EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
2	S	81.5	72.4	78.5	79.5	76.2	78.2	0	0	0	0	0	0
	P	75.0	70.0	75.0	76.3	75.3	77.0	0	0	0	0	0	0
	C	100	100	100	100	100	100	0	0	0	0	0	0
	C_1	100	100	100	100	100	100	0	0	0	0	0	0
4	S	80.0	78.8	78.8	78.4	79.2	77.5	0	0	0	0.5	0	0
	P	73.0	71.1	74.0	75.5	73.8	74.9	0	0	0	0.5	1.5	0.5
	C	100	100	100	100	100	100	0	0	0	0	0	0
	C_1	100	100	100	100	100	100	0	0	0	0	0	0
6	S	80.5	73.7	77.2	76.7	75.5	77.8	0	0	0	0	0	0
	P	74.2	72.6	70.5	75.4	73.3	74.8	0	0	0	0	0	0
	C	100	100	100	100	100	100	0	0	0	0	0	0
	C_1	100	100	100	100	100	100	0	0	0	0	0	0
8	S	80.3	75.5	70.3	76.0	74.5	77.3	0	0	0	0	0	0
	P	75.0	71.2	72.1	73.2	73.1	73.0	0	0	0	0	0.5	0
	C	100	100	100	100	100	100	0	0	0	0	0	0
	C_1	100	100	100	100	100	100	0	0	0	0	0	0
10	S	78.5	71.0	71.2	73.3	70.6	72.7	0	0	0.5	0	0	0
	P	70.1	68.3	70.5	70.0	70.2	71.3	0	0	0.5	0	0	0
	C	100	100	100	100	100	100	0	0	0	0	0	0
	C_1	100	100	100	100	100	100	0	0	0	0	0	0

4.6.6 Elimination of Overgrown Bacteria

The overgrown bacteria after co-cultivation was eradicated with the addition of cefotaxime. Washing with cefotaxime 200 mg l^{-1} resulted in 32.8 per cent explants with over growth of bacteria whereas after the second washing with cefotaxime 200 mg l^{-1}, only 0.8 explants were with bacteria (Table 36). Further increase in the concentration of cefotaxime at 250 and 300 mg l^{-1} also reduced the bacterial growth and percentage explants with overgrowth of bacteria after co-cultivation was 30.1 and 29.7 per cent respectively. After washing with 250 and 300 mg l^{-1} cefotaxime and in non-selective media the per cent explants with bacterial over growth was 0.3 and 0.1 respectively.

4.6.7 Effect of Acetosyringone

Among the different treatments, the maximum transformation efficiency of 3.0 was recorded with acetosyringone 100 μM in the plasmid PCAMBIA 2301 (EHA 105). The other treatments with 50, 150 and 200 μM acetosysingone did not produce 0.5 per cent transformants. The maximum transformation efficiency of 3.0 was also obtained with the plasmid PCAMBIA 2301 (EHA 105) in the control. Acetosyringone 100 μM recovered 0.5 per cent transformants in EHA 105 (PCAMBIA 1301), 1.5 percentage transformants in LBA 4404 (PCAMBIA 1301) and 2.5 percentage with LBA 4404 (PCAMBIA 2301). In the control transformants were obtained from the strains EHA 105 and LBA 4404 with PCAMBIA 2301 .

With 50, 150, 200 μM acetosyringone treatments the maximum transformation efficiency produced was only 0.5 per cent with the different vectors. So the total number of transformants isolated with 100 μM acetosyingone was maximum when compared to that in the other treatments.

Table 36 Effect of cefotaxime in the eradication of overgrown bacteria

Cefotaxime mgl^{-1}	Percent explants with overgrowth of bacteria											
	After co-cultivation						In induction medium					
	PBI 121		PCAMBIA 1301		PLAMBIA 2301		PBI 121		PCAMBIA 1301		PLAMBIA 2301	
	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
150	89.1	90.3	79.5	80.8	78.6	90.0	90.0	79.5	68.4	40.3	60.4	41.0
200	32.8	28.1	23.2	21.9	25.8	32.8	2.8	2.5	2.8	2.7	2.7	3.0
250	30.1	25.3	22.3	20.2	23.1	30.1	1.0	1.2	1.9	1.6	1.3	1.8
300	29.7	22.4	30.5	19.8	22.2	29.7	0.9	1.0	0.9	0.5	1.0	0.8
Control	100	100	100	100	100	100	100	100	100	100	100	100

4.6.8 Effect of Different Bacterial Strains

Among the two strains LBA 4404 and EHA 105 the maximum percentage of transformants (3.0) were obtained with EHA 105. With the vector pCAMBIA 1301 1.0 percentage transformants were obtained in LBA 4404 whereas 1.5 percentage transformants were obtained with EHA 105. With pCAMBIA 2301, 2.0 percentage transformants were obtained with LBA 4404 and 3.0 percentage with EHA 105.

4.7 GUS STAINING

Out of the total kanamycin resistant PLBs, 69.23 per cent showed GUS activity (Table.39). The X-gluc staining pattern varied among the PLBs from those fully stained to partially stained and stained in a particular location. The staining pattern also differed with the PLBs co-cultivated with different strains of *Agrobacterium viz.*, LBA4404 and EHA105 containing the vector pCAMBIA2301. The distribution of GUS expression (blue spots and blue patches) over the surface of the individual PLBs was even. In contrast to these observations, the PLBs from the non co-cultivated cells (control) did not stain blue.

4.8 PCR ANALYSIS

Amplification of DNA of all the four plants which stayed green and grew in the presence of kanamycin revealed the expected 0.7 kb fragment. Genomic DNA from four plants gave good PCR amplification. No amplification of the expected band was observed in the non-transformed control plant.

Table 37 Effect of Acetosyringone on transformation efficiency

Acetosyringone µM		Percent explants retained												Transformation efficiency					
		After co cultivation						After incubation											
		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301		PBI 121		pCAMBIA 1301		pCAMBIA 2301	
		EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404	EHA 105	LBA 4404
50	S	78.1	73.1	75.2	82.3	72.3	87.5	60.3	59.8	57.8	68.2	54.5	69.0	0	0	0	0	0	0
	P	62.3	60.8	63.6	65.4	61.9	65.0	45.8	48.2	49.9	50.8	43.1	47.2	0	0	0	0	0.5	0
	C_1	72.3	73.0	60.0	60.0	70.1	66.0	50.0	57.6	60.2	56.4	55.8	59.9	0	0	0	0	0	0
	C_2	70.0	61.1	58.0	62.2	62.0	62.8	48.3	45.5	60.5	50.3	40.1	50.3	0	0	0	0	0	0
	C_3	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0
100	S	79.4	78.4	79.9	84.0	80.5	88.4	60.2	63.1	61.4	67.9	61.4	70.3	0	0	0	1.0	1.0	1.0
	P	68.8	65.5	65.4	72.5	65.9	69.7	41.9	58.7	51.6	47.8	48.3	49.6	0	0	0.5	1.5	3.0	2.5
	C_1	70.0	75.2	80.2	85.0	77.8	80.1	55.4	65.2	61.4	65.4	60.4	70.0	0	0	0	0	1.0	0
	C_2	65.8	64.8	62.5	73.3	67.1	65.5	40.8	57.6	50.6	48.8	49.7	52.2	0	0	0	0	1.0	0.5
	C_3	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0
150	S	81.3	82.4	78.8	83.3	81.7	85.6	63.3	60.3	63.4	67.7	62.4	67.9	0	0	0	0	0.5	0
	P	72.2	74.3	66.0	71.5	67.2	65.3	51.7	54.8	58.9	53.6	49.9	48.5	0	0	0	0	0.5	0
	C_1	80.4	81.3	77.7	82.2	80.5	82.6	61.0	62.4	62.0	65.5	61.5	65.4	0	0	0	0	0.5	0
	C_2	72.3	75.0	65.2	70.5	69.9	63.3	51.0	55.0	57.8	52.4	50.0	49.9	0	0	0	0	0.5	0
	C_3	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0
200	S	70.4	71.4	75.3	80.6	80.2	81.4	55.0	57.2	62.8	63.4	60.7	65.4	0	0	0	0	0.5	0
	P	65.5	67.2	62.8	70.2	64.0	67.0	52.4	56.8	51.7	54.5	57.2	48.9	0	0	0	0	0	0
	C_1	70.2	70.0	71.	81.4	79.9	80.2	53.5	55.0	50.0	62.7	59.1	63.1	0	0	0	0	0.5	0
	C_2	62.2	60.2	60.4	69.2	65.5	65.0	51.0	53.8	49.8	52.1	55.4	48.0	0	0	0	0	0	0
	C_3	100	100	100	100	100	100	100	100	100	100	100	100	0	0	0	0	0	0

S - bacterial suspension (1 : 5 v/v) method

Control (C_1) - without acetosyringone (bacterial suspension)

P - Pellet method

C_1 - without acetosyringone (pellet method)

C_2 - control

Table 38 Transformation frequency and number of primary transformants obtained with different bacterial strains and plasmids.

Bacterial strains & plasmids		**Number of PLBs co-cultivated**	**Number of primary transformants obtained**	**Per cent transformants**
LBA4404	PBI 121	200	0	0
	pCAMBIA 1301	200	2	1.0
	pCAMBIA 2301	200	4	2.0
EHA105	pCAMBIA 1301	200	3	1.5
	pCAMBIA 2301	200	6	3.0
	PBI 121	200	0	0

Table 39. Transient *GUS* expression of *Dendrobium* PLBs

Explant	Plasmid and *Agrobacterium* strains	No. of explants stained	No. of *GUS* positives	Average number of spots/field
PLBs	LBA 4404 (pCAMBIA 1301)	3	1 (33.3)	1
PLBs	EHA 105 (pCAMBIA 2301)	13	9(69.23)	3
PLBs	LBA 4404 (pCAMBIA) 2301	3	2 (66.6)	1
PLBs	EHA 105 (PCAMBIA 1301)	3	2(66.6)	1
PLBs	Without cocultivation	3	Nil	Nil

4.9 TRANSFORMED PLANT

The height of one year old transformed plants ranged from 0.8 to 1.8 cm. The transformed plants produced 1-3 leaves in one year growth *in vitro* under stringent antibiotic selection conditions. The root length ranged between 0.5 to 1.5 cm and the leaf length from 0.5 to 1.1.cm (Table 40).

Table 40 Morphology of transformed plants

Treatments	Plantlet height (cm)	No. of leaves	Root length (cm)	Leaf length (cm)
Transformed T_1	1.0	1	1.0	1.1
T_2	0.8	2	0.5	0.8
T_3	1.7	2	1.2	1.0
T_4	1.5	2	0.9	0.5
T_5	1.8	3	1.5	0.5
Control	5.9	5	1.8	4.5

5. DISCUSSION

The genus *Dendrobium* is a renowned sympodial epiphytic orchid currently enjoying very high popularity among the commercial orchids. Many species of this second largest orchid genus produce very showy and attractive flowers of great ornamental value. *Dendrobium* is a widely cultivated cutflower in Kerala with much contribution to the agribusiness activity. The unique flower shapes and the long vase life contribute to the popularity of these flowers. There is an increasing demand for *Dendrobium* both in the domestic and international markets. Developing novel varieties for quality traits like flower colour, floral scent and increased vase life are essential for the cut flower industry. Further, new varieties will remain in high demand for a short period only. Evolving new varieties through traditional breeding is a time consuming process.

Molecular breeding, a supplement to traditional breeding is under way in cut flowers. It is being used for the improvement of flower crops the world over. Since floricultural and ornamental crops are grown for aesthetic and non-edible purposes, there is likely to be less concern in biosafety issues compared to other food crops. Hence there is considerable potential for developing transgenics in ornamental crops. Advances in transgenic technology provide new opportunities for manipulation of the genome. These will have significant impact on expanding and diversifying the gene pool of crop plants, introducing specific genes and shortening the time required for the production of new varieties or hybrids. Molecular breeding is beneficial to increase the production and quality by creating plants with enhanced resistance to diseases, insects or viruses and increased tolerance to environmental stresses like salinity, temperature or drought. Vegetative traits such as better growth habit, foliage and stem colour, adaptability to low light and successful environmental conditions may also be improved. Through these technique genes for shelf life, flower, colour

and architecture may be directly transferred so as to develop new varieties that are tailor made to customers preferences.

Among the different gene delivery systems available, *Agrobacterium tumefaciens* mediated one is the mostly widely used to introduce foreign genes into dicots (Weising *et al.*, 1988) and in some monocots (Eady *et al.,* 2000). Earlier *Agrobacterium* mediated transformation was considered difficult in monocotyledons. Recent advances in the understanding of the biology of the infections process, and the availability of gene promoters and selectable markers improved the progress of genetic transformation in monocotyledons (Smith and Hood, 1995). High frequency of transformation, broad host range, high rate of expression and stable integration of inserted genes have made *Agrobacterium* based gene transfer system the most popular one.

There are only a limited number of reports on the genetic transformation of *Dendrobium.* The initial reports were only in early nineties (Kuehnle and Sugii, 1992) and the particle bombardment method was mainly used for the transfer of genes. Only in recent past *Agrobacterium tumefaciens* is used to insert genes in *Dendrobium.*

The present study was undertaken at the Centre for Molecular Biology and Biotechnology, College of Agriculture, Vellayani to standardize the method of genetic transformation in *Dendrobium* through *Agrobacterium tumefaciens*. The various requirements for transformation were optimized so as to evolve a protocol.

The first requirement for molecular breeding is that the tissue to be genetically engineered must give rise to plants. In *Dendrobium* the protocorm derived from seeds, and the protocorm like bodies (PLB) derived from shoot apices are the tissues suitable for reliable plant production. For obtaining protocorms three-fourth mature *Dendrobium* pods were collected and subjected to various surface sterilization

treatments. Surface sterilization with mercuric chloride 0.08 per cent for ten minutes was the most effective. The percentage of aseptic cultures obtained with 0.08 per cent mercuric chloride was 80.8. With mercuric chloride 0.1 per cent, the survival percentage was low (45.9) due to browning and death of seeds. Mercuric chloride is the prefered surface sterilant for the crops grown in humid tropical conditions.

In order to improve the percentage of aseptic cultures this method was combined with alcohol dipping and flaming. It was observed that flaming the pods in two strokes, after dipping in alcohol, was an efficient treatment which reduced the contamination percentage. The percentage of aseptic cultures obtained by this method was 84.5. Over-ripe capsules were not suitable for flaming, as they burst during flaming. Lekharani (2002) also reported that harvesting pods after 90 per cent maturity resulted in the bursting of the over-ripe capsules during flaming, prior to inoculation.

Seed germination of the embryo culture, was observed after 50.6 days and subsequently protocorms were formed. The inherent genetic and physiological features were found to play a direct role in, *in vitro* seed germination. Nagashima (1993) studied the seeds of 47 orchid species and reported that the germination rate ranged from 0.8 to 100 per cent and the number of days from sowing to germination ranged from 3 to 305 depending on the stage of embryogenesis.

Inorder to establish PLBs, the shoot apices of two year old *Dendrobium* plants were collected, trimmed and subjected to various surface sterilization treatments. The surface sterilization with mercuric chloride 0.1 per cent ten minutes was found to be effective. The establishment percentage of aseptic cultures was 96.33. Mercuric chloride has been used by several workers and has been often observed to be more effective than sodiumhypochlorite (Sherly, 1997)

Use of coconut water was found beneficial, for the proliferation of PLBs. Coconut water 150 mll^{-1} in the PLB proliferation medium enhanced the proliferation rate and 26.01 PLBs were produced in eight weeks. When coconut water was not added, only 14.7 PLBs were produced. The coconut water contains kinetin, aminoacids, organic acids and vitamins. These nutrients might be responsible for the growth enhancement. Coconut water has been used by several workers and the beneficial effect of coconut water in growth enhancement has often been established (Morel ,1974; Bhasker ,1996; Lekharani,2002). The concentration of coconut water used was 10 to 15 per cent (Intowong and sagawa, 1973; Madhuri and Vasundhara, 1990).

Exudation of phenolic compounds into the culture medium was observed which subsequently led to tissue necrosis. Addition of activated charcoal 0.5gl^{-1} was found to be beneficial for the adsorption of phenolic compounds. With the addition of activated charcoal 0.5 gl^{-1} 27.9 PLBs were produced in eight weeks whereas only 15.5 PLBs were produced without the addition of charcod. Several workers viz., Fridborg *et al.*, (1978), Yam and Weatherhead (1988) had enumerated the beneficial effects of activated charcoal in the control of phenolics.

In order to increase the production of secondary and proliferating PLBs growth substances were combined with 150ml l^{-1} coconut water and 0.5g l^{-1} activated charcoal in the half-strength MS medium. A maximum of 28.3 PLBs were obtained with the addition of BA 0.2 mgl^{-1}. Several workers had observed that, the proliferation of PLBs *in vitro* was regulated by the interaction and balance between the growth substances supplied in the medium and produced endogenously by cultured cells (Shimasaki and Uemoto, 1987; Sherly 1997)

Selection of transformed cells is a key factor in developing a successful method of genetic transformation. Screening of transformed cells is very important; otherwise the untransformed cells would overgrow leading to the loss of transformed

cells. Normally the plant cells are sensitive to certain antibiotics and so the genes conferring resistance to those antibiotics are generally used a selectable markers. Once the plant is transformed with that antibiotic resistant gene and then if it is grown in the medium containing the concerned antibiotic, only the transformed cells can thrive. Only those transformed cells could multiply and grow normally, where as the untransformed ones are killed.

Several factors influence the efficiency of an antibiotic as a selection agent. The antibiotic used must be toxic to the plant cells; but the products from the dying, untransformed cells should not kill the adjacent transformed cells. Thus the most effective selection agents are those which either inhibit growth or slowly kill the untransformed tissues. Optimal selection pressure will use the lowest level of antibiotic needed to kill the untransformed tissues. All these factors necessitate the evaluation of sensitivity of plant tissue to different antibiotics and to select the optimum concentration of antibiotic that is required to inhibit growth. Hence, the sensitivity of both the protocorms an PLBs to different doses of ampicillin, rifampicin, cefotaxime, carbenicillin, kanamycin and hygromycin were evaluated.

It was observed that among the six antibiotics tested, the *Dendrobium* tissues were sensitive to kanamycin and hygromycin, at lower concentrations. Bleaching of protocorms and PLBs was observed with kanamycin 100mgl^{-1} from the sixth week of treatment. Kanamycin is a widely used marker for plant transformation and it is been used by several workers (Nagaraju *et al.*, 1998). Kanamycin was used at a strength of 100 to 200 mgl^{-1} for selection of *Dendrobium* transformants(Nan and Kuehnle, 1995 a). Yang *et al.* (1999) observed in *Cymbidium* that all the PLBs cultured on kanamycin 100 mgl^{-1} or above died 45 days after the subculture.

Protocorms and PLBs of *Dendrobium* showed total inhibition of growth at hygromycin 50 mgl^{-1}. Hygromycin had often been used in various crops. Hygromycin was used at a strength of 50 mgl^{-1} to select the transformants in

Phalaenopsis (Belarmino and Mii, 2000) and in African violet (Kushikawa *et al.*, 2001). Men et al. (2003) selected the *Dendrobium* transformants with hygromycin 30mgl^{-1}. This indicates that there is variation in the sensitivity/resistance to antibiotic depending on the genotype, physiological condition, size and type of explant and the tissue culture conditions (Yang et al., 1999). Both the protocorms and the PLBs are in active stage of growth and they have fast dividing cells.They therefore require more quantity of antibiotic to prevent further growth.

Elimination of bacteria from the plant tissues after co-cultivation is very important. Further, survival of bacteria in plant tissue may affect the growth and regeneration of transformed tissues. The bacterial elimination is done by using appropriate concentrations of antibiotics. Hence, there is a need to standardize the optimum concentration of a bacteriostatic agent. Cefotaxime 200 mgl^{-1} was used for the elimination of *Agrobacterium.* At this concentration, the orchid tissues remain unchanged. This concentration was selected as the optimum dose for the elimination of bacteria. Cefotaxime had been used successfully for the elimination of *Agrobacterium* in transformation work with a number of crops. It was successfully used at the strength of 200 mgl^{-1} in eliminating *Agrobacterium* from the inoculated explant during transformation in white clover (Voisey *et al.,* 1994). In casuarina, 250 mgl^{-1} strength of cefotaxime was used (Le *et al.*, 1996). In sedum, cefotaxime 300 mgl^{-1} (Yoon *et al.*, 2002), lavender 500 mgl^{-1} (Mishiba *et al.*, 2000) and in Africian violet 800 mgl^{-1} (Kushikawa *et al* ., 2001) was used.

Once an efficient *in vitro* regeneration protocol and the sensitivity levels of antibiotics have been developed, the next requirement for a successful genetic transformation is the selection of an efficient gene delivery system (Kuehnle, 1992*).* *Agrobacterium tumefaciens* mediated transformation was selected. Two strains of *Agrobacterium* viz., LBA 4404 and EHA 105 were used. There are differences in the susceptibility between species (Desgagnes et al., 1995) and even between cultivars

and genotypes of the species. Hence, transformation with different strains harbouring a good selectable marker was tried. As *Dendrobium* tissues were sensitive to kanamycin and hygromycin the vectors with kanamycin and hygromycin resistance were selected. The PBI 121 and pCAMBIA 2301 contain the kanamycin resistance gene and pCAMBIA 1301 harbour the hygromycin resistant gene as markers

Experiments were carried out to standardize the suitable size of the explant for the genetic transformation in *Dendrobium.* No transformant was obtained when protocorms were used as explants. Successful transformants (2.0 per cent) were isolated from the co-cultivation of PLBs of 0.2 cm size. When PLBs of 0.4 and 0.5 cm were co-cultivated, 0.5 percentage transformants were obtained. PLBs were used as explants for different transformation experiments in orchids. In *Dendrobium* (Yu *et al.,* 2001); *Cymbidium* (Yang *et al.,* 1999); *Brassia, Cattleya* and *Doritaenopsis* (Knapp *et al.,* 2000); *Phalaenopsis* (Chai *et al*., 2002); *Oncidium* (Liau *et al.,* 2003).

Successful transformants could be obtained from the co-cultivation of the PLBs. In the PLBs shoots developed directly from the primary and secondary meristems without an intervening organogenesis phase. This minimized the necessity of the treatment with phytohormones and thus the opportunity for somaclonal variation. When the PLBs were 1.0 mm in diameter, the newly formed PLBs were visible. PLBs contain more number of actively dividing cells. These cells were competent for transformation as reported by Braun (1975) and Iida *et al.* (1991) in tobacco. These cells were more susceptible to *Agrobacterium* infection. Thus greater the number of actively dividing cells in the explant to be infected with *Agrobacterium tumefaciens*, the higher was the probability of obtaining stable expression of the transformed gene.

An experiment was taken up to standardize the optimum number of PLBs that should be kept in a single petridish during the co-cultivation process. The number

of PLBs kept per petridish ranged between 20 and 60.Transformants could be obtained from the petriplates with 20 and 30 explants. In plates with more than 30 PLBs as the distance between two explants is less there was rapid spread of *Agrobacterium* which led to the overgrowth of the bacteria. Elimination of that overgrowth was very difficult and the co-cultivated PLBs were lost due to this bacterial overgrowth.

During the infection process, the *Agrobacterium* gets attached to the plant cell. Experiments were undertaken to standardize the optimum time required for the infection process. Among the different treatments, the explants immersed in bacterial suspension for 15 minutes gave the maximum percentage (2.5) of transformants. The length of the time that the explants were immersed in the *Agrobacterium* suspension influenced transformation. Several workers treated the plant tissues and the *Agrobacterium* suspension for a particular period of time and it varied according to the plant species and the bacterial strains. In *Gerbera* five minutes (Nagaraju *et al.*,1978) and in *Agapanthus* one minute (Suzuki *et al.*, 2001), and in *Cymbidium* 1.5 hours treatment (Chan *et al.*, 2003) resulted in transformants.

Wounding of PLBs with a scalpel was found beneficial. It was observed that wounding of PLBs helped in the recovery of maximum number of kanamycin (25.7) and hygromycin (30.2) resistant PLBs. Wounding the PLBs with a scalpel facilitated efficient infection of *Agrobacterium tumefaciens* to the explants. Generally in monocots co-cultivation with *Agrobacterium* requires tissue wounding. The maximum per cent transformants (2.0) were obtained with wounded explants. The friable nature of the *Dendrobium* PLBs made them susceptible to bacterial infection. Mishiba et al. (2000) observed that the friable nature of lavender callus made it susceptible to bacterial infection. Wounded cells release polyphenolic compounds like acetosyringone, which activate the *Agrobacterium vir* genes (Zambryski, 1992). When the *vir* genes were activated they facilitate the transfer of T DNA (which

contain the gene of interest) to the plant cell. Thus maximum transformants were obtained from the wounded PLBs. The wounded explants showed less bacterial overgrowth than the unwounded ones probably because of the good penetration of cefotaxime to the interior of the tissue. Homey (2000) also recorded similar observation in pepper.

Infection was carried out by two methods. The first method was by using pellets of bacterial cells, which were obtained on centrifugation of the overnight grown bacterial suspension. The second method was by using overnight grown bacterial suspension in 1:5 (v/v) dilution. Highest number (1.0) of transformants were recovered when the infection was carried out with pellets of bacterial cells. The same vector produced 0.5 percent transformants when the infection was carried out with 1:5 (v/v) dilution of overnight grown bacteria. The excess number of *Agrobacterium* in the pellets of bacterial cells might be responsible for the increased number of transformants. The number of *Agrobacterium* in the inoculum was a critical factor in the transformation of some species. In lettuce excessive number of bacteria stressed the plant cells (Michelmore et al. 1987). Alternatively if the number of *Agrobacterium* is low, the number of transformed cells is reduced. In *Datura*, a 1:10 or 1:20 (v/v) dilution of an overnight culture of bacteria, resulted in more transformed shoots compared to a 1:5 (v/v) dilution (Curtis *et al.,* 1999).

To standardize the number of days required for the co-cultivation, experiments were done. Transformants were obtained from the explants co-cultivated with *Agrobacterium* in dark for 2.0 and 3.0 days. No transformant was produced from the explants co-cultivated for more than three days. When co-cultivated for more than three days overgrowth of bacteria was noticed visibly. In a few petriplates a pinkish exudation was observed .The tissues were very soft and they broke down easily when touched with a sterile forceps. The tissues with such bacterial overgrowth were washed in liquid MS medium or sterile water with

cefotaxime 200 mgl^{-1} and then subcultured. This practice reduced the number of explants with bacterial overgrowth. After three washings the percentage of explants with overgrowth of bacteria was reduced to 3.0 per cent.

After bacterial infection and washing with cefotaxime to eliminate the excess bacteria after co-cultivation, the explants were blotted dry on sterile filter papers. The unblotted explants were lost due to contamination by fungal and bacterial pathogens. Complete elimination of bacteria from the explant after co-cultivation was essential. Otherwise it interfered with the growth and organogenesis of the explant, and resulted in the death of the explant and disrupted the experiment. Elimination of bacteria from the PLBs was done by the use of cefotaxime 200 mgl^{-1}. This antibiotic was chosen as it efficiently killed the bacteria, at the same time did not affect the growth of the PLBs. PLBs did not show any discoloration, bleaching or browning at this concentration. Cefotaxime was used by several workers for this bacterial eliminaton. Vergauwe *et al.* (1996) working on transformation of *Artemisia annua* found that cefotaxime at 50 mg l^{-1} was effective as a decontaminating antibiotic; but it caused retardation in callus formation. Then they tried vancomycin at 750 mg l^{-1} which was not toxic to the tissue. But it could not control the bacteria effectively. Belarmino and Mii (2000) used 300 mg^{-1} cefotaxime to eliminate the bacteria after co-cultivation of *Phalaenopsis* cell clumps with *A. tumefaciens.*

In spite of the commercial importance of this crop there are only a limited number of reports in the case of *Dendrobium*, a monocot. This may be because in monocot species such as rice and maize generally plant phenolic compounds like acetosyringone and hydroxy acetosyringone have not been produced in nature. Acetosyringone play a key role in the gene transfer process by *Agrobacterium. Agrobacterium tumefaciens* respond to certain plant phenolic compounds like acetosyringone and hydroxy acetosyringone. These small molecules act to induce activity of virulence *(vir)* genes that are encoded on the plasmid. The phenolic signal

molecules bind to the *Vir A* gene product, the *Vir A* protein. *Vir A* functions in conjugation with the protein *ChvE* (a sugar binding protein) which is located in the inner membrane of *Agrobacterium.* The *'Vir'* genes are located on 35Kb region of the plasmid that lies outside the TDNA region. When *A. tumefaciens* get attached to a plant cell, and the *'Vir'* genes are induced, then 'TDNA' (which contains the gene of interest) is transferred to plant cell. In monocots acetosyringone, the key factor for transformation is not present normally. *Dendrobium*, being a monocot it was assumed that acetosyringone was not secreted by nature. But in *Dendrobium* orchids the presence of an inducer of *A. tumefaciens* virulence genes have been confirmed by Nan *et al.* (1997). So the inducer of the *vir* gene would have been secreted and that favoured the infection of *A. tumefaciens* which generated 1.0 transformants in the control treatment without acetosyringone. Maximum transformation efficiency (3.0) was obtained with the addition of acetosyringone. So addition of acetosyringone in the co-cultivation medium and in the bacterial suspension might have acted synergistically to increase the infection and the transformation efficiency.

Several workers had used acetosyringone at the concentration of 20 to 200 μM to generate transformed plants. In *Gladiolus* (Babu and Chawla, 2000); in lavender (Mishiba *et al.*, 2000);in *Phalaenopsis* (Belarmino and Mii., 2000), (Chan *et al.*, 2003); and in *Agapanthus* (Suzuki *et al.,* 2001) acetosyringone was added to obtain transformants.

Four weeks after bacterial infection (subculturing at 10 days interval), the co-cultivated and non-co-cultivated cells bleached on selection medium containing kanamycin 200 mgl^{-1} and hygromycin 100 mgl^{-1} depending on the vector PBI 121 pCAMBIA 2301, and pCAMBIA 1301. At third subculture the transformed cells turned to light green colour while the non transformed cells turned brown and died. Fresh sprouts initiated on these light green colour PLBs. Screening of transformed cells was done by several workers by using various antibiotics according to the

antibiotic resistant genes in the vector. Kuehnle and Sugii (1992) identified the potentially transformed tissues of *Dendrobium* by the growth and green colour on half-strength MS medium with 50-100 mg l^{-1} kanamycin sulphate. Kanamycin concentrations that prevented growth of non transformed tissues could not be used for long term selection because such levels suppressed the regeneration of potentially transformed tissues. Levels of 100-200 mg l^{-1} kanamycin were later found adequate for *Dendrobium* by Nan and Kuehnle (1995). Kanamycin 100 μg/ml was used by Hsieh *et al.* (1997) and 100 mg l^{-1} by Babu and Chawla (2000) to select the transformed plants of *Phalaenopsis* and *Gladiolus*, respectively.

Yu *et al.* (1999) and Belarmino and Mii (2000) identified the transformed tissues of *Cymbidium* and *Phalaenopsis* with 50 mgl^{-1} hygromycin . Kushikawa *et al.* (2001) in African violet, and Kim *et al.* (2002) selected the *Alstroemeria* transformants with 50 and 20 mgl^{-1} hygromycin. In *Phalaenopsis* Chai *et al.* (2002) carried out selection with 3 mgl^{-1} hygromycin. Transgenic *Gerbera* (Korbin *et al.*, 2002), Sedum (Yoon *et al.*, 2002) and Carnation (Lin *et al.*, 2003) were selected with kanamycin 7,25, and 25 mgl^{-1} respectively. Boase *et al.*, (2002) selected the transformed cells of *Cyclamen* with kanamycin 50 mgl^{-1}

There were differences in the transformation efficiency with respect to the different strains of *Agrobacterium.* Maximum percentage (3.0) of transformants were obtained with the strain EHA 105. In the vector pCAMBIA 1301, the transformants obtained with LBA 4404 and EHA 105 were 1.0 and 1.5 per cent respectively. With pCAMBIA 2301, 2.0 and 3.0 per cent transformants were obtained with LBA 4404 and EHA 105. Thus differences in the transformation efficiency according to the bacterial strains was evident. This is because; the *Agrobacterium* infectivity is a result of the interaction between the plant cell and the bacterial cell. The infectivity is improved by the use of right strain of the bacteria, varying host genotype, manipulating explant physiology, inoculation and

co-cultivation conditions (Godwin *et al.,* 1992) Agrobacterium mediated transformation has been reported to be dependent on *Agrobacterium* strain, conditions of co-cultivation, selection method and mode of regeneration (Mathis and Hinchee, 1994)

Confirmation of transformants is very important. Histochemical Gus assay and PCR analysis were often used by workers to confirm the transgenic (Nan and Kuehnle, 1995, Yang *et al.,* 1999; Yoon *et al.,* 2002).

The PBI 121, pCAMBIA1301 and pCAMBIA2301 harbour the *gus* gene as repoter gene. Some reporter gene products can be detected in intact plant tissues. The most popular of these systems is the *E.coli* β-D-glucuronidase (GUS) gene. It encodes a stable enzyme that is not normally present in plants and that catalyzes the cleavage of a range of β-D-glucuronides. Thus the GUS activity in transformed plant tissues can be localized by observing the blue colour that is formed after the hydrolysis of the uncoloured substrate 5-bromo4-chloro3-indolyl β-D-glucuronic acid. Further confirmation was done by PCR analysis.

Among the antibiotic resistant PLBs, 69.23 per cent showed GUS activity. GUS activity was detected in more than 50 per cent PLBs. As not all the cells of PLBs produce plants, the calculation of roughly one third of those PLBs with transient GUS activity may serve as a maximum approximation of PLB with potential to produce stable transformants (Nan and Kuehnle, 1995). The X gluc staining pattern varied among the PLBs from those fully stained to partially stained and stained in a particular site. The staining pattern also differed with the PLBs cocultivated with different strains of *Agrobacterium viz.,* LBA4404 and EHA105 containing the vector (pCAMBIA2301). The distribution of GUS expression (blue spots and blue patches) over the surface of the individual PLBs was even. In contrast to these observations the PLBs from the non co-cultivated cells (control) did not

stain. Kanamycin selection screens the relatively rare stable integration events, from high transient activity levels. Transgenic PLBs survived the antibiotic screening whereas untransformed PLBs were effectively killed. Among those PLBs selected by antibiotic screening, only a few were GUS negatives. It is possible that the GUS negatives among the selected shoots may be the escapes (Yang *et al.,* 1999). The same workers observed that some GUS negative plants turned out to be positive in the PCR analysis for *npt* II gene. Yoon *et al.* (2002) reported that out of the total kanamycin resistant shoots, only 2.5 per cent and in *Cyclamen* Boase *et al.* (2002) reported that 47 per cent were positive for GUS. In Carnation (Lin *et al.*, 2003) around 55 per cent of the plants and in *Phalaenopsis* (Chan *et al.,* 2003) 100 per cent showed GUS activity (Lin *et al.,* 2003). Liau *et al.* (2003) obtained 28 independent transgenic *Oncidium* from which six were positive for β-glucuronidase

Variation in the transgene expression level in *Dendrobium* was observed by the variation in intensity and distribution of the blue staining with the GUS assay and the degree of colour change in PLBs. This is in conformity to the findings of Trifonova *et al.* (2001). Young proliferating PLBs regenerated from the putative transgenics gave consistent GUS expression while older PLBs stained exclusively along the wounded edge. Satyavathi *et al.* (2002) also observed similar GUS staining pattern in cotton and it is evident that the young and tender tissues showed good expression of GUS activity. Histochemical GUS expression is consistent in actively dividing cells.

DNA was isolated from the transformed and the control (untransformed) plants. DNA samples were amplified by PCR with the *gus, npt* and *hpt* primers designed for *gus, npt* and *hpt* genes. The amplified products were run on gel electrophoresis. DNA from transformed plants of *Dendrobium* were found by PCR analysis to contain a distinct band for 0.7 kb *nptII* gene fragment. The 1.2kb *Uid*

A gene fragment was not noticed. Integration of transgene was confirmed by PCR by several workers. Nan and Kuehnle (1995) also observed a distinct band for 0.7 kb *nptII* gene fragment. Several reasons may account for what appears to be a high level of *nptII* expression. First, a dosage effect may arise from gene duplication and / or multiple insertions. PCR analysis was used to confirm the transformants in rose (Li *et al.*, 2002), sedum (Yoon *et al.*, 2002), coleus (Bauer *et al.*, 2002), petunia (Shaw *et al.*, 2002) and chrysanthemum (Seo *et al.*, 2003). PCR analysis verified the presence of the *npt II* gene in the plant DNA isolated from antibiotic resistant shoots (Taskin *et al.*, 2003).

The transformed plants were maintained in continuous antibiotic selection for one year. So the growth was reduced by more than 50 per cent compared to the plants on non-selective medium. Kuehnle and Sugii (1992) and Belarmino and Mii (2000) also observed that of slow growth of the transformed plants compared to the untransformed ones. In the present study the height of the transformed plants ranged from 0.8 to 1.8 cm. The transformed plants produced 1-3 leaves in one year growth *in vitro.* The root length ranged between 0.5 to 1.5 cm and the leaf length from 0.5 to 1.1 cm. According to Kuehnle and Sugii (1992), the one year old transformed plants of *Dendrobium* had healthy green leaves and green roots with total lengths ranging from 0.5 to 2.9 cm after the first year on selection medium.

Molecular breeding of *Dendrobium* is very exciting due to the wealth of new orchid varieties that may be created. During this study various techniques for genetic transformation in *Dendrobium* were optimized. Type of explants, size of explants, co-cultivation time, infection time, incubation time, acetosyringone concentration, selection agents *etc.* were optimized. Based on the results of this study several areas are identified, as requiring particular attention for successful *Dendrobium* transformation. Also there exist some inherent problems associated with the *in vitro* response of orchids which needs further investigations. In the present study the

transgenic tissues exhibited a very slow growth rate @ 8 PLBs of 4mm size were formed in 45 days of *in vitro* culture, where as 25 PLBs of 4mm size were formed in 45 days by the untransformed PLBs. The growth was reduced by more than 50 per cent compared to untransformed plants. The assessment of sensitivity to antibiotics needs longer time (more than 6 weeks) in *Dendrobium* when compared to other crops. *Dendrobium* is poorly sensitive to certain aminoglycosides like kanamycin which is commonly used for selection of transformants. The sensitivity to aminoglycosides like kanamycin could be observed only in the sixth week. So all these factors necessitate long period for selection of the transformed tissues. Owing to these limitations, in a commercially important crop like *Dendrobium* the transformation technique, would be of much significance. In this study a maximum of 3.0 per cent transformants where obtained. The transformants were confirmed by GUS histochemical assay and PCR analysis. In *Dendrobium*, a monocot, which is not sufficiently subjected to genetic transformation, this study provided a protocol for successful genetic transformation. Further improvement of transformation efficiency can be made possible by adopting certain techniques like addition of '*Vir*' gene, inducing compounds (stimulants), use of various promoters, alternate selection agents or positive selection agents etc. *Dendrobium* orchids with our optimised conditions of explant selection, co-cultivation, infection, incubation and screening procedures can be effectively engineered to produce desirable traits. By using these improved transformation procedures, genes for modifying the floral architecture, precocious flowering, improved flower colour, size, fragrance, extended vase life and viral resistance can be engineered into *Dendrobium.*

6. SUMMARY

A study on *Agrobacterium* mediated genetic transformation techniques in *Dendrobium*, Sonia 17 was carried out in the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani during 2002 to 2004. The salient findings of the above studies are summarised in this chapter.

Surface sterilization of the pods with mercuric chloride 0.08 per cent for ten minutes was found to be the most effective treatment. Sterlization by flaming the pods in burner after dipping in 70 per cent ethanol was also tried. Mercuric chloride (0.08 per cent) treatment together with two stroke flaming resulted in rapid initiation of protocorms (50.62 days).

Effect of different doses of coconut water on germination and number of days taken for protocorm initiation was studied. Out of the six treatments coconut water 150 mll^{-1} proved to be the best interms of per cent germination (84.84) and early initiation of protocorms (50.62 days).

The studies revealed that the treatment with activated charcoal 0.5 gl^{-1} recorded the maximum germination per cent.

Surface sterilization of the shoot tips with 0.10 per cent mercuric chloride for ten minutes was found to be the best, with the maximum establishment (96.33%).

Significant difference was observed among the different concentrations of coconut water on the per cent establishment and the proliferation rate of PLBs. Out of the various treatments, the maximum establishment (95.0%) and the maximum proliferation rate (26.01) were observed with coconut water 150 ml l^{-1}.

Effects of different doses of activated charcoal on the establishment and proliferation rate of PLBs were studied. The maximum establishment (87.5%) and

the maximum proliferation rate (27.9) were observed with activated charcoal 0.5 mgl^{-1}.

The plant growth substance BA 0.2 mgl^{-1} supplemented in the PLB establishment medium could induce maximum number of PLBs (28.3) in eight weeks.

Experiments were conducted to evaluate the sensitivity of both protocorms and PLBs to different doses of antibiotics.

Increased doses of ampicillin 400 to 500 mgl^{-1} caused bleaching of protocorm tissues, and ampicillin 450 and 500 mgl^{-1} induced bleaching of the PLBs. However, no browning and death were observed at higher concentrations of amplicillin 450 and 500 mgl^{-1}, even after eight weeks.

In the seventh and eighth week, bleaching was observed in protocorms with rifampicin 400 mgl^{-1} and browning and death with rifampicin 450 and 500 mgl^{-1}. With PLBs, increased doses of 450 and 500 mgl^{-1} induced only bleaching.

Increased doses of cefotaxime 450 to 500 mgl^{-1} induced browning of the PLBs in the eighth week. The protocorms in cefotaxime 350 to 500 mgl^{-1} turned brown in the seventh and eighth week.

High concentration of carbenicillin from 350 to 500 mgl^{-1} induced browning of the protocorms and PLBs in carbenicillin 450 to 500 mgl^{-1} turned brown.

Bleaching of protocorms and PLBs was noticed with kanamycin 100 mgl^{-1} from the sixth week onwards. Protocorms and PLBs were bleached at 50 mgl^{-1} hygromycin. It was infered that out of the six antibiotics, kanamycin 200 mgl^{-1} and hygromycin 100 mgl^{-1} can be used as selection agents for screening the transformants.

Effect of cefotaxime in eliminating the growth of *Agrobacterium* was studied. It was observed that the *Agrobacterium* was effectively killed by cefotaxime 200 mgl^{-1}.

The *Agrobacterium tumefaciens* produced white slimy and smooth colonies. The strain LBA 4404 harbouring, PBI 121, pCAMBIA 1301 and pCAMBIA 2301 took 38.0, 35.0 and 30.8 hours respectively for growth with colonies.

The *Agrobacterium tumefaciens* strain EHA 105 harbouring the binary plasmids PBI 121, pCAMBIA 1301 and pCAMBIA 2301 showed growth with colonies in 36.5, 30.0 and 29.9 hours respectively.

When protocorms were used as explants none of the transformants were obtained in all the binary plasmids PBI 121, pCAMBIA 1301 and pCAMBIA 2301.

The maximum of 2.0 percentage transformants were obtained with EHA 105 harbouring pCAMBIA 2301 when the PLBs of 0.2 cm were co-cultivated. The transformation efficiency of 0.5 per cent was obtained when the PLBs of 0.4 and 0.5 cm size were co-cultivated.

It was observed that in the experiment carried out with 20 explants per petriplate the transformants (0.5) were obtained only with the infection of explants with pellets. When 30 and 40 explants were kept per petridish (90mm) transformation efficiency was from (0.5 to 1 per cent). When 50 explants were kept in a petriplate, overcrowding occurred and the infection spread to the nearby explants and overgrowth of bacteria was noticed. This overgrowth was dense and it was not possible to eradicate the overgrown bacteria with the addition of cefotaxime.

The transformation efficiency was the highest when the infection was carried out with pellets of bacterial cells (1.0). The explants infected with the bacterial suspension recorded only 0.5 percentage success.

The infection was carried out for 10, 15 and 20 minutes so as to standardize the optimum time required for infection. Among the different treatments, transformants were observed only with 15 minutes infection period. In the 15 minutes infection period 1.0 per cent transformants were obtained with the infection process by using the bacterial pellets. Infection by 1:5 (v/v) dilution produced 0.5 percentage transformants.

When the infection was done with bacterial pellets the wounded PLBs showed the best response with a recovery of maximum number of resistant PLBs (20.1) compared to the unwounded (5.3).

Among the wounded PLBs the per cent PLBs resistant to kanamycin (PBI 121, pCAMBIA 2301) and hygromycin (pCAMBIA 1301) were high (18.9) when compared to (1.1) the infection carriedout with bacterial suspension 1:5 v/v.

Among the different treatments, transformants were obtained from the explants co-cultivated with *Agrobacterium* in the dark for 2.0 and 3.0 days. No transformant was produced from the explants co-cultivated for more than three days.

The maximum transformation efficiency (2.0) was obtained when LBA 4404 (pCAMBIA 1301) and EHA 105 (pCAMBIA 2301) were co-cultivated for three days and the infection was carried out with bacterial pellets.

The maximum transformation efficiency of 3.0 per cent was recorded with the addition of acetosyringone 100μM.

Out of the total kanamycin resisted PLBs, 69.23 per cent were positive for GUS activity. The distribution of GUS expression (blue spots and blue patches) was

observed over the surface of individual PLBs. The antibiotic sensitive PLBs did not stain.

Amplification of DNA of the four plants which stayed green and grew in the presence of kanamycin revealed the expected 0.7 kb fragment. Genomic DNA from four plants gave good PCR amplification. No amplification of the expected band was observed in the control.

The height of the one year old transformed plants ranged from 0.8 to 1.8 cm. They produced 1 – 3 leaves during the one year growth *in vitro* under stringent antibiotic selection conditions. The root length ranged between 0.5 and 1.5 cm and the leaf length from 0.5 to 1.1 cm.

7. REFERENCES

Abdulkarim, A.G. and Hairani, H.1992. Plantlet production from young leaves of *Mokara*. *Lindleyana* 7 : 11 - 12

Agarwal, D.C., Morwal, G.C. and Mascarenhas, A.F. 1992. *In vitro* propagation and slow growth storage of shoot cultures of *Vanilla walkeriae* Wight an endangered orchid. *Lindleyana* 7 : 95 - 99

An, G., Evert, P.R., Mitra, A. and Ha, S.B. 1988. *Plant Molecular Biology Manual*. (ed. Gelvin, S.B and Schilperoort, R.A.). Kluwer Academic Publishers. Dordrecht, Netherlands, pp 1 - 19

An, G., Watson, B.D. and Chiang, C.C. 1986. Transformation of tobacco, tomato, potato and *Arabidopsis thaliana* using Ti vector system. *Plant Physiol.* 81 : 301-305

Anzai, H., Ishii, K., Nojiri, C., Morikawa, H. and Tanaka, M. 1995. Transformation of *Phalaenopsis* using particle bombardment. *Orchid Biology : Reviews and Perspectives* (eds. Arditti, J and Pridgeon, A.M.). Kluwer Academic, London, pp. 75 -115

Arditti, J. 1967. Factors affecting the germination of orchid seeds. *Bot. Rev.* 33 : 1-97

Arditti, J. 1979. Aspects of orchid physiology. *Advances in Botanical Research* Vol 7 (eds. Woolhouse, H.W.). Academic Press, New York, pp. 421 - 655

Arditti, J. 1992. *Fundamentals of Orchid Biology*. John Wiley and Sons, New York, 691 p.

Arditti, J. and Ernst, R.1993. *Micropropagation of Orchids*. John Wiley and Sons, New York, 682 p.

Babu, P. and Chawla, H.S. 2000. *In vitro* regeneration and *Agrobacterium* mediated transformation in gladiolus. *J. Hort. Sci. Biotech.* 75 : 400 - 404.

Bauer, N. Levanic, D.L., Mihaljevic, S. and Jelaska,S. 2002. Genetic transformation of *Coleus blumei* Benth using *Agrobacterium*. *Food Tech. Biotech.* 40 : 163 - 169

Belarmino, M.M. and Mii, M. 2000. *Agrobacterium* mediated genetic transformation of a *Phalaenopsis* orchid. *Plant Cell Rep.* 19 : 435 - 442.

Bellard, W.W. 1987. Sterile propagation of *Cypripedium reginae* from seeds. *Am. Orchid. Soc. Bull.* 56: 935 - 945

Bernard, G.R. and Jack, P.I. 2001. Molecular Biotechnology –Second Edition. ASM Press, Washington, D, C. 683 p.

Bewan, M., Flavel, R.B. and Chilton, M.D. 1983. A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature.* 304 : 184 -187

Bhaskar, J.1996 Mircopropagation of *Phalaenopsis*. Ph.D thesis, Kerala Agricultural University, Thrissur, India, 207 p.

Boase, M.R., Marshal, G.B., Peters, T.A. and Bendall, M.J. 2002. Long term expression of the *gus A* reporter gene in transgenic *Cyclamen* produced from etiolated hypocotyl explants. *Plant Cell Tissue Organ Cult.* 70: 27 – 39

Braun, A.C. 1975. The cell cycle and tumorigenesis in plants. *Cell.Differ.* 7 : 177 - 196

Chai, M.L., Xu, C.J., Senthil, K.K., Kim, J.Y. and Kim, D.H. 2002. Stable transformation of protocorm like bodies in *Phalaenopsis* orchid mediated by *Agrobacterium tumefaciens*. *Scientia Hort.* 96 : 213 - 224

Chan, Y.L., Chen, W.H. and Chang, M.T. 2003. *Phalaenopsis* orchid gene transformation I. Optimization of transient gene expression. *J. Chinese. Soc. Hort. Sci.* 49 : 33 – 44

Chen, F.C. and Kuehnle, A.R. 1996. Obtaining transgenic *Anthurium* through *Agrobacterium* mediated transformation of etiolated internodes. *J. Am. Soc. Hort. Sci.* 121 : 47 – 51

Chen, L., Hatano, T. and Niimi, Y. 2002. High efficiency of *Agrobacterium* mediated rhizome transformation in *Cymbidium*. *Lindleyana.* 17: 130 - 134

Chen, P.Y., Wang, C.K., Soong, S.C., and To, K.Y., 2003. Complete sequence of the binary vector PBI121 and its application in cloning T-DNA insertion from transgenic plants. *Mol. Breed.* 11:287 - 293

Chia, T.F., Chan, Y.S. and Chua, N.H. 1994. The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. *Plant J.* 6 : 441 - 446

Churchill, M.E., Ball, E.A. and Arditti, J. 1970. Production of orchid plants from seedling leaf tips. *Orchid Digest.* 271 - 273

Churchill, M.E., Ball, E.A. and Arditti, J. 1972. Tissue culture of orchids II Methods for root tips. *Am. Orchid. Soc. Bull.* 41 : 726 - 730

Condylife, P.G., Devey, M.R., Power, J.B., Koehorst, V.H. and Visser, P.B. 2003. An optimised protocol for rose transformation applicable to different cultivars. *Acta Hort.* 612 : 115 - 120

Curtis, I.S., Power, J.B., Hedden, P., Ward, D.A., Phillips, A., Lowe, K.C. and Davey, M.R. 1999. A stable transformation system for the ornamental plant *Datura meteloides. Plant Cell Rep.* 18 : 554 - 560

De Block, M., De Brouwer, D. and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica Oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. *Plant Physiol.* 91 : 694 - 701

Dekeyser, R., Claes, B., Marichal, M., Van Montagu, M. and Captan, A.1989. Evaluation of selectable markers for rice transformation. *Plant Physiol.* 90 : 217 - 223

Devi, J., Nath, M. and Deka, P.C. 1990. Effect of different media on germination and growth of some North East Indian species of *Dendrobium. J. Orchid Soc. India.* 4: 45-49

Dodds, J.H. and Roberts, L.W. 1982. Experiments in Plant Tissue Culture. Cambridge University Press, London, 178 p.

Dohm, A., Ludwig, C., Schilling, D. and Debener, T. 2002. Transformation of roses with genes for antifungal proteins to reduce their susceptibility to fungal diseases. *Acta Hort.* 572 : 105 - 111

Dunemann, F., Illgner, R.and Stange, I. 2002. Transformation of *Rhododendron* with genes for abiotic stress tolerance. *Acta Hort.* 572. 113 - 120

Desgagnes, R., Laberge, S., Allard, G., Khoudi, H., Castonguary, Y., Lapointe, J., Michaud, R. and Vezina, L.P. 1995. Genetic transformation of Commercial breeding lines of alfalfa (*Medicago sativa*). *Plant Cell Tissue Organ Cult.* 42 : 129 - 140

Eady, C.C., Welf, R.J and Lister, C.E. 2000. *Agrobacterium tumefaciens* mediated transformation and transgenic plant regeneration of onion (*Allium cepa* L.). *Plant Cell Rep.* 19: 376 - 381

Ellis, D., Roberts, D., Sutton, B., Lazaroof, W., Webb, D. and Flinn, B. 1989. Transformation of white spruce and other conifer species by *Agrobacterium tumefaciens*. *Plant Cell Rep.* 8 : 16 - 20

Ernst, R. 1974. The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum*. *Am. Orchid Soc. Bull.* 43 : 35 - 38

Ernst, R., Arditti, J. and Healey, P.L.1971. Carbohydrate physiology of orchid seedlings. II Hydrolysis and effects of oligosaccharides. *Am. J. Bot.* 58 : 827 - 835

Fonnesbech, M. 1972. Growth hormones and propagation of *Cymbidium in vitro*. *Physiol. Plant.* 27 : 310 - 316

Fridborg, G., Pedersen, M., Landstrom, L.E. and Eriksson, T.1978. The effects of activated charcoal on tissue cultures : Adsorption of metabolites inhibiting morphogenesis. *Physiol. Plant* 33 : 104 - 106

Fu, F.M.L. 1978. Clonal propagation of *Aranda, Ascocentra and Cattleya* by leaf tissue culture. *Gardens Bull.* 31 : 132 - 138

George, A.J. 1997. Micropropagation of *Dendrobium* (*D. ostenholt*). M.Sc. thesis, University of Agricultural Sciences, Bangalore, India, 68 p.

George, E.F. and Sherington, P.D. 1984. Plant propagation by Tissue Culture. Eastern Press, 690 p.

Giovannini, A., Morreale, G., Berio, T., Mascarello, C. and Allavena, A. 2002. Modification of flowering time in *Osteospermum ecklonis*, L. by CONSTANS gene. *Acta Hort.* 572 : 163 - 167

Godwin, I.D., Ford Lloyd, B.V. and Newbury, H.J. 1992. *In vitro* approaches of extending the host range of *Agrobacterium* for plant transformation. *Aust. J. Bot. 40* : 751 – 763

Goh, C.J. 1970. Tissue culture of *Vanda* Miss Joaquim. *J. Natl. Acad. Sci.* 2: 31 - 33

Griesbach, R.J. 1994. An improved method for transforming plants through electrophoresis. *Plant Sci.* 102: 81 - 89.

Hajdukiewicz, P., Svab. Z. and Maliga. P. 1994. The small, versatile PzP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* 25 (6) : 989 - 994

Harrison, C.R. and Arditti, J. 1978. Physiological changes during the germination of *Cattleya aurantiaca* (Orchidaceae) *Bot. Gaz.* 139 : 180 -189

Harvais, G.1972. The development and growth requirements of *Dactylorrhiza purpurella* in asymbiotic cultures. *Can. J. Bot.* 50 : 1223 - 1229

Hazarika, R.B. and Sarma, C.M 1995. *In vitro* germination and regeneration of *Dendrobium transparens* Lindl. *J. Orchid Soc. India.* 9 : 51 - 54

Hew, C.S. and Math, T.C. 1989. Sugar uptake and invertase activity in *Dendrobium* tissues. *New Phytologist.* 111 : 161 - 171

Hew, C.S., Ting, S.K. and Chia, T.F. 1988. Substrate utilization by *Dendrobium* tissues. *Bot. Gaz.* 149 : 153 - 157

Hinnen, M.G.J., Pierik, R.L.M and Bronsema, F.B.F. 1989. The influence of macronutrients and some other factors on growth of *Phalaenopsis* hybrid seedlings *in vitro*. *Scientia Hort.* 41 : 105 - 116

Homey, C. 2000. *Agrobacterium* mediated transformation in black Pepper *(Piper nigrum L.)*. Ph.D. thesis, Kerala Agricultural University, Thrissur, 128 p.

Honjo, T., Futaya, Y. and Takakura. 1988. Effect of Co_2 enrichment on the growth of *Cymbidium* PLB *in vitro*. *Acta Hort.* 230 : 185 - 188

Hsieh, R.M., Chen, W.H., Hsu, H.M., Lin, Y.S., Tsai, W.T., Fu, Y.M., Chan, M.T. and Yu, S.M. 1997. *Agrobacterium tumefaciens* mediated transformation of *Phalaenopsis* orchid. *Taiwan Sugar Res.* 44 : 12 - 18

Ichihashi, B. and Kako, S. 1977. Studies on the clonal propagation of *Cattleya* by tissue culture. II Browning of *Cattleya*. *J. Jap. Soc. Hort. Sci.* 46 : 325 - 330

Ichihashi, S. 1990. Effects of light on root formation of *Bletilla striata* seedlings. *Lindleyana.* 5 : 140 - 143

Ichihashi, S. 1992. Micro propagation of *Phalaenopsis* through the culture of lateral buds from young flower stalks. *Lindleyana* 7 : 208 - 215

Iida, A., Yamashida, T., Yamada, Y., and Morikawa. H., 1991. Efficiency of particle bombardment mediated transformation is influenced by cell stage in synchronized cultured cells of tobacco. *Plant Physiol.* 97 : 1585 - 1587

Intuwong, O. and Sagawa, Y. 1973. Clonal Propagation of Sarcanthine orchids by aseptic culture of inflorescence. *Am. Orchid Soc.Bull.* 42 : 209 - 215

Irawati, S.S., Suseno, H. and Idris, S. 1977. Tissue culture of *Aranthera* James Storie. *Orchid Rev.* 85 : 138 - 142

Irifune, K., Morimoto, Y. and Uchihama, M. 2003. Production of herbicide resistant transgenic lily plants by particle bombardment. *J. Jap. Soc. Hort. Sci.* 72 : 511 - 516

Ito, I.1955. Germination of seeds from immature pod and subsequent growth of seedlings in *Dendrobium nobile* Lindl. *Scient. Rep. Saikyo Univ. Agric.* 7 : 35 - 42

Jefferson, R.A., Kavanagh, T.A., and. Bewan. M.W., 1987. *GUS* fusions: *B glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6 : 3901 - 3907

Jonojit, R. and Nirmalya, B. 2003. Induction of callus and plant regeneration from shoot tip explants of *Dendrobium fimbriatum.* Lindl. *var Oculatum. Scientia Hort.* 97: 333 - 340

Jordan, M.R.1965. Seed flasking with steam sterilization. *Am. Orchid Soc. Bull.* 34 : 219

Kamo, K. and Blowers, A. 1999. Tissue specificity and expression level of *gus A* under *rol D*, mannopine synthase and translation elongation factor 1 subunit ∝ promoters in transgenic *Gladiolus* plants. *Plant Cell Rep.* 18 : 809 - 815

Kim, J.B., Jeu, M.J., Raemakers, C.J.J.M., Jacobsen, E and Visser, R.G.F. 2002. The effect of hygromycin on regeneration in different *Alstroemeria* explants types after *Agrobacterium* mediated transformation. *Acta Hort.* 572 : 97 - 103

Kishimoto, S., Aida, R. and Shibata, M. 2002. *Agrobacterium tumefaciens* mediated transformation of Elatior Begonia (Begonia X Liemalis) Fotsch. *Plant Sci.* 162 : 697 - 703

Knapp, J.E., Kausch, A.P. and Chandlee, J.M. 2000. Transformation of three genera of orchid using the bar gene as a selectable marker. *Plant Cell Rep.* 19 : 893 - 898

Knudson, L. 1946. A new nutrient solution for the germination of orchid seeds. *Am. Orchid Soc. Bull.* 15 : 214 - 217

Knudson, L. 1951. Nutrient solution for orchids, *Bot. Gaz.* 112 : 528 - 532

Komari, T. and Kudo, T. 1999. Methods in genetic transformation, *Agrobacterium tumefaciens*, *Molecular improvement of cereal crops* (eds. Vasil, I.K.). Kluwer Academic Publishers, Dordrecht pp. 43 - 83

Korbin, M., Podwyszynska, M., Komorowska, B. and Wawrzynczak, D.2002. Transformation of gerbera plants with tomato spotted wilt virus (TSWV) nucleoprotein gene. *Acta Hort.* 572 : 149 - 157

Kotomori, S. and Murashige, T. 1965. Some aspects of aseptic propagation of orchids. *Am. Orchid Soc. Bull.* 34 : 484 - 489

Krieg, L.C., Walker, M.A., Senaratua, T. and Mckersie, B.D. 1990. Growth, ammonia accumulation and glutamine synthetase activity in alfalfa *(Medicago sativa)* shoots and cell cultures treated with phosphinothricin. *Plant Cell Rep.* 9 : 80 – 83

Krishnan, P.N., Latha, P.G. and Seeni, S.1993. Biochemical changes during protocorm formation from *in vitro* grown embryos of *Spathoglottis plicata* Blume. *J. Orchid Soc. India.* 7 : 87 - 91

Kudo, S., Shibata, N., Kanno, Y. and Suzuki, M.2002. Transformation of *Chrysanthemum (Dendranthema grandiflorum)* via *Agrobacterium tumefaciens. Acta Hort.* 572 : 139 - 147

Kuehnle, A.R. 1997. Molecular biology of orchids. *Orchid Biology – Review and Perspectives. VII* (eds Arditti, J. and Pridgeon, A.M.). Kluwer Academic, London, pp. 75 – 115

Kuehnle, A. R and Sugii, N. 1992. Transformation of *Dendrobium* Orchid using particle bombardment of protocorms. *Plant Cell Rep.* 11: 484 - 488

Kukulczanka, K and Wojciechowska, U. 1983. Propagation of two *Dendrobium* species by *in vitro* culture. *Acta Hort.* 131 : 105 - 110

Kumaria, S. and Tandon, P. 1991. Asymbiotic germination of *Dendrobium fimbriatum* var *oculatum* seeds on different media. *Biol. Sci.* 57:277 - 279

Kushikawa, S., Hoshino, Y. and Mii, M.2001. *Agrobacterium* mediated transformation of *Saintpaulia ionantha* Wendl. *Plant Sci.* 161 : 953 - 960

Kusumoto, N. 1979. Effect of combination of growth regulators and of organic supplements on the proliferation and organogenesis of *Cattleya* PLB's cultured *in vitro. J. Jap. Soc. Hort. Sci.* 47 : 502 – 510

Kusumoto, M. 1980 Effects of coconut milk, agar and sucrose concentrations and medium pH on the proliferation of *Cymbidium* protocorm like bodies cultured *in vitro. J. Jap. Soc. Hort. Sci* 48 : 503 - 509

Le,Q.V., Bogusz, D., Gherbi, H., Lappartient, A., Duhoux, E and Franche, C. 1996. *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca* a tropical nitrogen fixing tree. *Plant Sci.*118 : 57 - 69

Lee, Y.I. and Lee, N. 2003. Plant regeneration from protocorm derived callus of *Cypripedium formosanum*. *In vitro Cell Dev. Biol.* 39 : 475 - 479

Lekharani, C. 2002. Intra and interspecific hybridization in *Dendrobium* Spp. Ph.D Thesis, Kerala Agricultural University, Thrissur, 360 p.

Li, X.G., Krasnyanski, S. F. and Korban, S.S. 2002. Optimisation of the U*id* A gene transfer into somatic embryos of rose via *A. tumefaciens. Plant Physiol. Biochem.* 40 : 453 - 459

Liau, C.H., You, S.J., Prasad, V., Hsiao, H.H., Lu, J.C., Yang, N.S and Chan, M.T. 2003. '*Agrobacterium tumefaciens* mediated transformation of an *Oncidium* Orchid. *Plant Cell Rep.* 21 : 993 - 998

Lin, R.C., Chen, L.Q, Bao, M. and Sun, Z.Y. 2003. Establishment of efficient *Agrobacterium tumefaciens* mediated genetic transformation system for carnation *(Dianthus caryophyllus)*. *For. Res.* 16 : 123 -128

Madhuri, S. and Vasundhara, G. 1990. Micro propagation of *Dendrobium* Joannie Ostenhault. *J. Orchid Soc. India. 4 : 145 - 148*

Mathews, V.H. and Rao, P.S 1980. *In vitro* multiplication of *Vanda* hybrids through tissue culture technique. *Plant Sci. Lett.* 17 : 383 - 389

Mathews, V.H and Rao, P.S 1985. *In vitro* culture of *Vanda* hybrid (Vanda TMA x Vanda Miss Joaquim) I Studies on protocorm explants. *Plant Sci. Lett.* 22 : 96 - 103

Mathis, N.L. and Hinchee, M.A.W.1994. *Agrobacterium* inoculation techniques for plant tissues. *Plant Molecular Biology Manual* (eds. Gelvin, S.B. and Schilperoort, R.A). Kluwer Academic Publishers, Dordrecht, pp. 1 - 9

Mc Intyre, D.K., Veitch, G.J. and Wrigley, J.W. 1974. Australian orchids from seed. II. Improvement in techniques and further successes. *Am. Orchid Soc. Bull.* 43: 52 - 53

Men, S., Ming, X., Wang, Y., Liu, R., Wei, C. and Li, Y. 2003. Genetic transformation of two species of orchid by biolistic bombardment. *Plant Cell Rep.* 21: 592 - 598

Mercuri, A., Benedetti, L., De Bruna S., Breg liano, R., Binachini, C., Foglia, G. and Schiva, T.2003. *Agrobacterium* mediated transformation with r*ol* genes of L*ilium longiflorum* Thunb. *Acta Hort.* 612: 129 - 136

Mercuri, A., Sacchetti, A., Benedetti, L.D., Schira, T. and Alberti, S. 2001. Green fluorescent flowers. *Plant Sci.* 161 : 961 – 968

Michelmore, R.W., Marsh, E., Seely, S. Landry, B. 1987. Transformation of lettice (*lactuca sativa)* mediated by *Agrobacterium tumetaciens*. *Plant Cell Rep.* 6 : 439 - 442

Mishiba, K.I., Ishikawa, K., Tsujii, O.and Mii. M., 2000. Efficient transformation of lavender (L*avandula latifolia*) mediated by *Agrobacterium. J. Hort. Sci. Biotech.* 75: 287 - 292

Mitra, G.C.1971. Studies on seeds, shoot tips and stem discs of an orchid grown in aseptic culture. *Indian J. Exp. Biol.* 9: 79 - 85

Mitra, G.C., Prasad, R.N and Roychowdhury, A.1976. Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. *Indian. J. Exp. Biol.*14: 350 - 351

Mondal, T.K. and Chand, P.K. 2000. Detection of genetic variation among Micropropagated tea (*Camellia sinensis*) by RAPD analysis. *In Vitro Cell Dev .Biol.* 38 :296-299

Moore, C.R. and Tripepi, P.R. 2003. Preconditioning *Rhododendron* and *Chrysanthemum* leaf explants affects transient β- glucuronidase expression after biolistic particle bombardment. *Acta Hort*. 625 : 363 - 369

Morbel., J., Jager, U., Wetzel, T., Krczal, G. and Feldhoff, A. 2002. Transformation of *Osteospermum ecklonis* (Cape daisy) with lettuce mosaic poly virus derived constructs. *Acta Hort*. 568 : 155 - 158

Morel, G.M. 1965. Clonal propagation of orchids by meristem culture. *Cymb. Soc. Newsl.* 20 : 3 - 11

Morel, G.M. 1974. Clonal multiplication of Orchids. (eds.C. Withner). Scientific Wiley Interscience, New York, pp. 169 - 222

Mosich, S.K., Ball, E.A. and Arditti, J. 1974. Clonal propagation of *Dendrobium* by means of node cultures. *Am. Orchid. Soc. Bull.* 43: 1055 – 1061

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473 - 496

Nagaraju, V., Srinivas, G.S.L. and Sita, G.L. 1998. *Agrobacterium* mediated genetic transformation in *Gerbera hybrida. Curr. Sci.* 74: 630 - 634

Nagashima, T. 1993 Studies on relationship between embryogenesis and germination in Orchidaceae. *J. Jap. Soc. Hort. Sci.* 62: 581 - 594

Nan, G.L. and Kuehnle, A.R. 1995. Genetic Transformation in *Dendrobium*. *Biotechnology in Agriculture and Forestry*, Vol 34, Plant protoplasts and Genetic Engineering VI, (eds. Bajaj, Y.P.S.). Springer Verlag, New York, pp.149 -160

Nan, G.L. and Kuehnle, A.R. 1995a. Factors affecting gene delivery by particle bombardment of *Dendrobium* orchids. *In Vitro Cell Dev. Biol.* 31 : 131 - 136

Nan, G.L., Tang, C.S., Kuehnle,A.R. and Kado,C.I. 1997. *Dendrobium* orchids contain an inducer of *Agrobacterium* virulence genes. *Physiol. Mol. Plant Pathol.* 51 : 391 – 399

Nath, M., Devi, J., Borthakur, B., Sharma, J. and Deka, P.C. 1991. Embryo culture of *Rhyncostylis retusa* and *Vanda coerulea.J.Orchid Soc. India* 5 : 97 - 101

Nehra, N.S., Chibbar, R.N., Kartha, K.K., Datla, R.S.S., Crosby, W.L. and Stushnoff, C. 1990. Genetic transformation of strawberry by *Agrobacterium tumefaciens* using a leaf disc regeneration system. *Plant Cell Rep.* 9 : 293 - 298

Nitsch, J.P 1969. Experimental androgenesis in *Nicotiana. Phytomorphology* 19 : 389 - 404

Paek, K.Y. and Yeung, E.C. 1991. The effect of Naptnalene acetic acid and N – Benzyl adenine on the growth of *Cymbidium forestii* rhizomes *in vitro. Plant Cell Tissue Organ Cult.* 29 : 65 - 71

Pathak, P., Vij, S.P. and Mahant, K.C. 1992. Ovule culture in *Goodyera biflora* : A study *in vitro. J. Orchid Soc. India.* 6 : 49 - 53

Pawlicki, N., Sangwan, R.S. and Norreel, B.S. 1992. Factors influencing the *Agrobacterium tumefaciens* mediated transformation of carrot *(Daucus carota L.). Plant Cell. Tissue Org. Cult.* 31 : 129 - 139

Pierik, R.L.M., Sprenkels, P.A., Hurst, B.V.D and Meys, Q.G.V.D. 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. i*n vitro. Scientia Hort.* 34: 139 - 153

Prasad, R.N. and Mitra, G.C. 1975. Nutrient requirements for germination of seeds and development of protocorms and seedlings of *Cymbidium* in aseptic cultures. *Indian J. Exp. Biol.* 13 : 123 - 126

Puddephat, I.J., Thompson, N., Robinson, H.T., Sandhu, P. and Henderson, J., 1999. Biolistic transformation of broccoli *(Brassica Oleracea)* for transient expression of the *B – glucuronidase* gene. *J. Hort. Sci. Biotech.* 74: 714 - 720

Puontikaertas, J., Stabel, P. and Eriksson, T. 1987. Transformation of Pea *(Pisum sativum L.).* by *Agrobacterium tumefaciens. Plant Cell Rep.* 8 : 321-324

Pyati, A.N and Murthy, H.N. 1995. *In vitro* seed germination and seedling development of *Dendrobium ovatum* (Willd.) Kranzl. *J. Orchid Soc. India. 9 :* 69 - 74

Raghavan, V and Torrey, J.G .1964. Inorganic nitrogen nutrition of the seedlings of the Orchid. *Cattleya. Am. J. Bot.* 51 : 264 - 274

Raghuwanshi, A.N., Mishra, R.R. and Sharma, G.D. 1986. Effect of pH on asymbiotic seed germination and seedling development of orchids. *Biology, Conservation and Culture of Orchids* (ed. Vij, S.P.). The orchid society of India, India, pp. 453 - 462

Rajmohan, K. 2003. Application of biotechnology in the improvement of ornamentals. *Proceedings of National Symposium on recent advances in Indian Floriculture, 12 - 14 Nov, 2003*. (eds. Rajeevan, P.K., Valsalakumari, P.K. and Misra, R.L.). Kerala Agricultural University, Thrissur, Kerala, pp. 185 - 189

Ram, M.S.N. and Mohandas,S. 2003. Transformation of African Violet *(Saintpaulia ionantha* with Glucanase chitinase genes using *A. tumefaciens. Acta Hort.* 625 : 471 - 478

Rao, A.N. and Avadhani, P.N. 1964. Some aspects of *in vitro* culture of *Vanda* seeds. *Gardens Bull.* 17 : 194 - 202

Rashid, H., Toriyama, K. and Hinata, K. 1996. Transgenic plant production from leaf discs of *Moricandia arvensis* using *Agrobacterium tumefaciens. Plant Cell Rep.* 15: 799 - 803

Reddy, P.V., Nanjan, K. and Shanmugavelu, K.G. 1992. *In vitro* studies in tropical orchids : Seed germination and seedling growth. *J. Orchid Soc. India* 6 : 75 - 78

Reyburn, A.N. 1978. The effect of pH on the expression of a darkness requiring dormancy in seeds of *Cypripedium reginae* wall. *Am. Orchid Soc. Bull.* 47 : 798 - 802

Rosa, M.D. and Laneri, U.1977. Modification of nutrient solutions for germination and growth *in vitro* of some cultivated orchids and for the vegetative propagation of *Cymbidium cultivars. Am. Orchid Soc.Bull.* 46: 813 - 820

Rosati, C., Simoneau, P., Treutter, D., Poupard, P., Cadot, Y., Cadic, A. and Duron, M.2003. Engineering of flower colour in *Forsythia* by expression of two independently transformed dihydroflavonol 4 reductase and anthocyanidin synthase genes of flavonoid pathway. *Mol. Breed.* 12 (3) : 197 - 208

Rubulo, A., Chavez, V. and Martinez, A.1989. *In vitro* seed germination and reintroduction of *Bletia Urbana* (Orchidaceae) in its natural habitate. *Lindleyana* 4 : 68 - 73

Sagawa, Y. and Sehgal, O.P. 1967. Aseptic stem propagation of *Vanda* Miss Joaquim. *Pacific Orchid Soc. Bull.* 25 : 17 - 18

Sagawa, Y. and Shoji, T. 1967. Clonal Propagation of *Dendrobiums* through shoot meristem culture. *Am. Orchid. Soc. Bull.* 36 : 856 - 859

*Sahid, C.A. 1980. Seed germination of a *Dendrobium* hybrid on several culture media. *Bull. Penelitian Hortikultura* 6 : 577 - 590

Sangama, 1986. Studies on *in vitro* seed germination and morphogenesis in orchids. Ph.D thesis, University of Agricultural Sciences, Bangalore, India, 152 p.

Satyayathi., V.V., V. Prasad, B. Gitalakshmi, and G. Lakshmi Sita. 2002. High efficiency transformation protocol for three Indian Cotton varieties Via *Agrobacterium tumefaciens*. *Plant Sci.* 162: 215-23.

Scott, R.J. and Arditti, J. 1959. *Cymbidium* from pod to pot. *Am. Orchid Soc. Bull.* 28 : 823 – 829

Seo, S., Choi, D.C., Kim, J.M., Lim, H.C., Kim, H.J., Choi, J. and Choi, Y.2003. Plant regeneration from leaf explants and efficient *Agrobacterium* mediated transformation system of *Chrysanthemum (Dendranthema grandiflorum). Acta Hort.* 625 : 403 - 409

Sharma, S.K. and Tandon, P. 1990. Asymbiotic germination and seedling growth in *Cymbidium elegans* L. and *Coelogyne punctulata* L. as influenced by different carbon sources. *J. Orchid Soc. India* 4 : 149 - 159

Sharon, M., Bagde, B. and Bagde, P.1992. Regeneration potential of wound infected *Dendrobium* Snowfire protocorms. *J. Orchid Soc. India,* 6 : 55 - 57

Shaw, J., Chen, H., Tsai, M., Kwo, C.I, Huang, L., 2002. Extended flower longevity of *Petunia hybrida* plants transformed with *boers*, a mutated ERS gene of *Brassica oleracea. Mol. Breed.* 9: 211 - 216

Sherly, K. 1997. Standardisation of in vitro techniques for mass multiplication of *Aranthera* and *Dendrobium.* Ph.D thesis, Kerala Agricultural University, Thrissur, 207 p.

Shimamoto, K., Terada, R., Izawa, T. and Fujimoto, H. 1989. Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature*. 338 : 274 - 276

Shimasaki, K. and Uemoto, S. 1987. Studies on the micro propagation of Japanese *Calanthe* species. *Sci. Bull. Faculty Agrl. Kyushu University* 42 : 9 - 14

Shinoyama, H., Komano, M., Nomura, Y. and Nagai, T. 2002. Introduction of delta endotoxin gene of *Bacillus thuringiensis to Chrysanthemum (Dendranthema grandiflorum)* Kitumura for insect resistance. *Breed Sci.* 52 : 43 - 50

Shinoyama, H., Mochizuki, A., Komano, M., Nomura, Y. and Nagai, T. 2003. Insect resistance in transgenic chrysanthemum. (*Dendranthema* X *grandiflorum* kitamura) by the introduction of a modified ∂- endotoxin gene of *Bacillus thuringiensis. Breed. Sci.* 53 : 359 - 367

Singh, F. 1993 *In vitro* Orchid seed germination and cloning of orchids - A success story. Plant Biotechnology: Commercial prospects and problems (eds. Prakash, J. and Pierik, R.L.M). Oxford and IBH Publishing Company, New Delhi, pp.85 - 109

Singh, F.1992. *In vitro* propagation of Orchids, 'State of the art'. *J Orchid Soc. India* 6: 11 - 14

Smith, R.H. and Hood, E.E. 1995. *Agrobacterium tumefaciens* transformation of monocotyledons. *Crop Sci.* 35 : 301 - 309

Soediono, N. 1983. Use of coconut water, NAA, 2,4, D and vitamins in shoot tip cultures of *Dendrobium* cv Jacqueline Thomas White. *Orchid Rev.* 91 : 86 - 87

Soediono, N. 1988. Use of coconut water, NAA, 2, 4, D and vitamins in shoot tip culture of *Dendrobium* cv Jacquelin Thomas white. *Orchid Rev*. 96 : 86 - 87

Steinhart, W.L., Winkler, R.J., Brierley, K.L., Hickman, M. J and Merlis, D.T. 1997. Expression of the β – glucuronidase reporter gene from a variety of promoter constructs in *Cattleya* protocorms and their protoplasts. *Lindleyana.* 12: 172 - 179

Stewart, J. and Button, J. 1975. Tissue culture Studies in paphiopedilum. *Am. Orchid Soc. Bull.* 44 : 591 - 598

Suzuki, S., Kanyaratt, S., Masahiro, M., Masam, N., 2001. Production of transgenic plants of the Liliaceous ornamental plant *Agapanthus praecox* SSP. *Orientalis* (Leighton) Leighton via *Agrobacterium* mediated transformation of embryogenic calli. *Plant Sci.* 161 : 89 - 97

Taskin, K.M., Turgut, K., Ercan, A.G. and Scott, R.J. 2003. *Agrobacterium* mediated tansformation of *Arabis gunnisoniana. Plant Cell Tissue and Org. Cult.* 72 : 173 - 179

Tee, C.S., Marziah, M., Tan, C.S. and Abdullah, M.P. 2002. Evaluation of different promoters driving the GFP report genes and selected target tissues for particle bombardment of *Dendrobium* Sonia 17. *Plant Cell Rep.* 21 : 452 - 458

Teo, C.K.H., Kunisaki, J.T and Sagawa, Y. 1973. Clonal propagation of Strap leaved *Vanda* by shoot tip culture. *Am. Orchid Soc. Bull.* 42 : 402 - 405

Torres, A.C., Cantliffe, D.J., Laughner, B., Bimiek, M., Nagata, R., Ashraf, M. and Ferl, R.J. 1993. Stable transformation of lettuce cultivar South Bay from cotyledon explants. *Plant Cell Tissue Org. Cult.* 34 : 279 - 285

Trifonova, A., S. Madsen, and A. Olesen, 2001. *Agrobacterium* mediated transgene delivery and integration into barley under a range of *in vitro* culture conditions. *Plant Sci.* 161 : 871 - 880

Vacin, E and Went, F.W 1949. Some pH changes in nutrient solutions. *Bot.Gaz.* 110 : 605 - 613

Vajrabhaya, M. and Vajrabhaya, T. 1970. Tissue Culture of *Rhyncostylis g[illegible]ntea*, a monopodial orchid. *Am. Orchid. Soc. Bull.* 39 : 907 - 910

Veluthambi, K., Aditya Gupta, K. and Sharma, A. 2003. The current status of plant transformation technologies . *Curr. Sci.* 84 : 368 – 380

Vergauwe, A., Cammaert, R., Vandenberghe, D., Genetello, C., Inze, D., Van Montagu, M., Van Den Eeckhout, E. 1996. *Agrobacterium tumefaciens* mediated transformation of *Artemisia annua* L. plants and the regeneration of transgenic plants. *Plant Cell Rep.* 15: 929 - 933

Vergauwe, A., Van Geldre, E., Inze, D., Van Montagu, M. and Van Den Eeckhovt, E. 1998. Factors influencing *Agrobacterium tumefaciens* mediated transformation of *Artemisia annua* L. *Plant Cell Rep.* 18 : 105 - 110

Villolobes, L. and Munoz, A.M.1994. Tissue culture of Orchids. *Cattleya*, *Encyclia, Oncidium* and *Stanhopea. Orchid Rev.* 102 : 58 - 62

Voisey, C.R., White, D.W.R., Dudas, B., Appleby, R.D., Eaung, P.M. and Scott, A.G. 1994. *Agrobacterium* mediated transformation of white clover using direct shoot organogenesis. *Plant Cell Rep.* 13 : 309 - 314

Weising, K., Schell, J and Kahl, G.1988. Foreign gene in plants : transfer, structure, expression and application *Ann Rev. Genet.* 22 : 421 - 477

White, D.W.R and Greenwood, D.1987. Transformation of the forage legumes *Trifolium repens* L. using binary *Agrobacterium* vectors. *Plant Mol. Biol.* 8 : 461 – 469

Xiang, Y., Yu, F. and Peng, Z.H. 2003. Tissue culture of *Cymbidium sinensis. For. Res.* 16 : 434 – 438

Yam, T.W. and Weatherhead, M.A. 1988. Germination and seedling development of some Hong Kong Orchids. *Lindleyana.* 3 : 156 – 160

Yam, T.W., Ernst, R., Arditti, J., Helen Nair and Weatherhead, M.A.1990. Charcoal in orchid seed germination and tissue culture media. *Lindleyana.* 5 : 256 - 265

Yang, J., Lee, H.J., Shin, D.H., Oh, S.K., Seon, J.H., Paek, K.Y. and Han, K.H. 1999. Genetic transformation of *Cymbidium* orchid by particle bombardment. *Plant Cell Rep.* 18 : 978 - 984

Yoon, E.S., Jeong, J.H and Choi, Y.E. 2002. Recovery of Basta resistant *Sedum erythrostichum* via *Agrobacterium* mediated transformation. *Plant Cell Rep.* 21 : 70 - 75

Yu, H., Yang, S.H. and Goh, C.J. 2001. *Agrobacterium* mediated transformation of a *Dendrobium* orchid with the class 1 knox gene DOH1 *Plant Cell Rep.* 20 : 301 - 305

Yu, Z.H., Chen, M.Y., Nie, L., Lu, H.F., Ming, X.T., Zheng, H.H., Qu, L.J. and Chen, Z.L. 1999. Recovery of transgenic orchid plants with hygromycin selection by particle bombardment of protocorms. *Plant Cell Tissue Organ Cult.* 58 : 87 – 92

Zambryski, P.C. 1992. Chronicles from the *Agrobacterium* plant cell DNA transfer story. *Annu. Rev. i 'nt Physiol. Plant Mol.Biol.* 43 : 465 – 490

Zhang, J.Y., Yu, L.F. and Lian, H.K. 1993. Some factors influencing the growth and differentiation of *Cymbidium goeringii* protocorms. *Plant Physiol.* 29 : 175 – 178

* Originals not Seen

AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION IN *DENDROBIUM*

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Abstract of the thesis submitted in partial fulfillment of the requirement for the degree of

Doctor of Philosophy in Horticulture

Faculty of Agriculture
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2005

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ABSTRACT

A study on '*Agrobacterium* mediated genetic transformation' in *Dendrobium*, Sonia 17 was conducted in the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani during 2002 – 2004.

Protocorms were obtained from the *in vitro* germination of *Dendrobium* seeds on half – strength MS medium supplemented with coconut water 150 ml l^{-1}, sucrose $30gl^{-1}$ and agar 6 gl^{-1}. Protocorm like bodies were produced from the *in vitro* culture of the shoot apices on half-strength MS medium with coconut water $150mgl^{-1}$, activated charcoal, 0.5 gl^{-1}, sucrose $30gl^{-1}$, agar $6gl^{-1}$ and BA 0.2 gl^{-1}.

Experiments were conducted to evaluate the sensitivity of both protocorms and PLBs to different doses of antibiotics *viz.*, ampicillin, rifampicin, cefotaxime, carbenicillin, kanamycin and hygromycin. It was observed that ampicillin 400 and 450 mgl^{-1} induced complete bleaching of the protocorms and PLBs, respectively. In the seventh and eight week, bleaching was observed in protocorms with rifampicin 400 mgl^{-1}, and browning and death with rifampicin 450 mgl^{-1}. With PLBs none of the treatments showed browning and death. Even increased doses of 450 mgl^{-1} induced only bleaching. Increased doses of cefotaxime 450 mgl^{-1} induced browning of the PLBs and cefotaxime 350 mgl^{-1} caused the browning of the protocorms in the eighth week. Bleaching was observed with carbenicillin 350 mgl^{-1}. Carbenicillin 450 mgl^{-1} induced browning and subsequent death of PLBs. Bleaching of protocorms and PLBs was noticed with kanamycin 100 mgl^{-1} from the sixth week onwards. Total inhibition of the growth of the protocorms and PLBs was observed with hygromycin 50 mgl^{-1}. Out of the six antibiotics used the orchid tissues were highly sensitive to kanamycin 100 mgl^{-1} and hygromycin 50 mgl^{-1}.

Effect of cefotaxime for the elimination of *Agrobacterium* was studied. It was observed that *Agrobacterium* was effectively killed by cefotaxime 200 mgl^{-1}.

Two strains of *Agrobacterium viz.,* LBA 4404 and EHA 105 were used for the experiments. The strain LBA 4404 habouring PBI 121, pCAMBIA 1301 and pCAMBIA 2301 took 38.0, 35.0 and 30.8 hours respectively for growth. The strain EHA 105 harbouring the binary plasmids PBI 121, pCAMBIA 1301 and pCAMBIA 2301 showed growth in 36.5, 30.0 and 29.9 hours respectively.

When the protocorms were used as explants, none of the transformants were obtained in all the binary plasmids PBI 121, pCAMBIA 1301 and pCAMBIA 2301. The maximum of 2.0 percentage transformants were obtained when the PLBs of 0.2 cm were co-cultivated. When the PLBs of 0.4 and 0.5 cm size were co-cultivated. 0.5 per cent transformants were obtained. The per cent exp. ts (98.2) retained after co-cultivation was maximum when the explants were infected by bacterial suspension. But the transformation efficiency was the highest 1.0 in the pelleting method. Overcrowding occured when 50 explants were kept in a petriplate during co-cultivation which led to the overgrowth of the bacteria. (With 20, 30, and 40 explants the transformation efficiency of 0.5, 0.5 and 1 per cent was obtained.)

In a study conducted to optimize the time required for infection, transformants (1.0 per cent) were obtained with the infection process for 15 minutes using the bacterial pellets. The infection done by diluting the bacterial suspension to 1:5 (v/v) produced 0.5 per cent transformants. Wounding was found beneficial and 18.9 kanamycin and hygromycin resistant PLbs were obtained when compared to unwounded explants (1.1), when the infection was done with bacterial suspension diluted to 1:5 v/v. With the infection using the bacterial pellets the wounded PLBs showed best response and maximum of 20.1 antibiotic resistant PLBs were obtained where as only 5.3 PLBs were recovered with the unwounded explants.

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Transformants were recovered only with the wounded PLBs of 0.2 cm size co-cultivated in the dark for 2.0 and 3.0 days. None of the transformants were produced from the explants co-cultivated for more than three days. The maximum transformation efficiency of 2.0 per cent was obtained when LBA 4404 (pCAMBIA 1301) and EHA 105 (pCAMBIA 2301) were co-cultivated for three days and the infection was carried out with the bacterial pellets. The loss of explants due to fungal contamination was avoided by blotting the explants in a blotting paper before inoculation. This practice helped in the retention of 80.3 per cent explants whereas only 30.1 per cent explants were retained after co-cultivation when the explants were not blotted. The transformation efficiency was increased to 3.0 per cent when acetosyringone 100 μM was added.

Out of the total kanamycin resistant PLBs 69.23 per cent were positive for GUS activity. The GUS expression was observed as blue spots and blue patches on the surface of the PLBs. The antibiotic sensitive PLBs did not stain. Amplification of DNA of the four plants which stayed green and grew in the presence of kanamycin revealed the expected 0.7 kb fragment. Genomic DNA from those plants gave good PCR amplification. No amplification of the expected band was observed in the control. The height of the transformed plants ranged from 0.8 to 1.8 cm. They produced 1-3 leaves in one year growth *in vitro* under stringent antibiotic selection conditions. The root length ranged between 0.5 to 1.5 cm and the leaf length from 0.5 to 1.1 cm.

APPENDIX – I

Basic Chemical Composition of the Media Employed for *In vitro* Culture

Chemical	**Quantity (mg/l)**
Major elements	MS
$CaCl_2.2H_2O$	440
$FeSo_4.H_2O$	27.8
KNO_3	1900
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$(NH_4)_2\ SO_4.2H_2O$	500
$Na_2.EDTA.2H_2O$	37.3
Minor elements	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
H_3BO_3	6.2
KI	0.83
$MnSO_4$	22.30
$Na_2MOO_4.2H_2O$	0.25
$ZnSO_4$	8.6

Organic Constituents

Glycine	2.0
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose g/l	30.0
Agar g/l	6.0
Inositol	100

APPENDIX II

Composition of the media employed for the culture of *Agrobacterium*

YEP medium (per l)

Yeast extract	-	10g
Peptone	-	10g
Sodium chloride	-	5.0g
P^H	-	7.0

AB Minimal medium

AB Salts		g/l
Ammonium Chloride	-	20
Magnesium Sulphate	-	6
Potassium Chloride	-	3
Calcium Chloride	-	0.2
$FeSO_4.7H_2O$	-	0.05
P^H	-	7.0
AB Buffer		
K_2HPO_4	-	60g
NaH_2PO_4	-	20g

(Prepare both AB Salts and Buffer, autoclave and store)

AB Glucose

Glucose 0.5g (autoclave and store)

Agar	-	1.5

APPENDIX III

Modified Mondal, Singh and Ahuja's Method

Leaves were ground to fine powder using liquid nitrogen

↓

Hot (65°C) extraction buffer and polyvinyl pyrollidone (PVP) were added to it

↓

Slurry of grounded plant material was transferred to extraction buffer kept in water bath at 65°C for 20 minutes

↓

Lysate was squeezed through four layers of sterile muslin cloth

↓

Chloroform : Isoamyl Alcohol (24 : 1) extraction was done

↓

To the supernatent, two volume of cold absolute ethanol and $1/10^{th}$ volume Of 3.0 M sodium acetate were added

↓

DNA was precipitated

↓

70 per cent ethanol wash was given

↓

DNA was stored in Tris Hcl-EDTA buffer

PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Books

Search Nucleotide for

Limits Preview/Index History Clipboard Details

GenBank all to file

Range: from begin to end Reverse complemented strand Features: SNP CDD MGC HPRD

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  JOURNAL   Mol. Breed. 11, 287-293 (2003)
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  TITLE     Direct Submission
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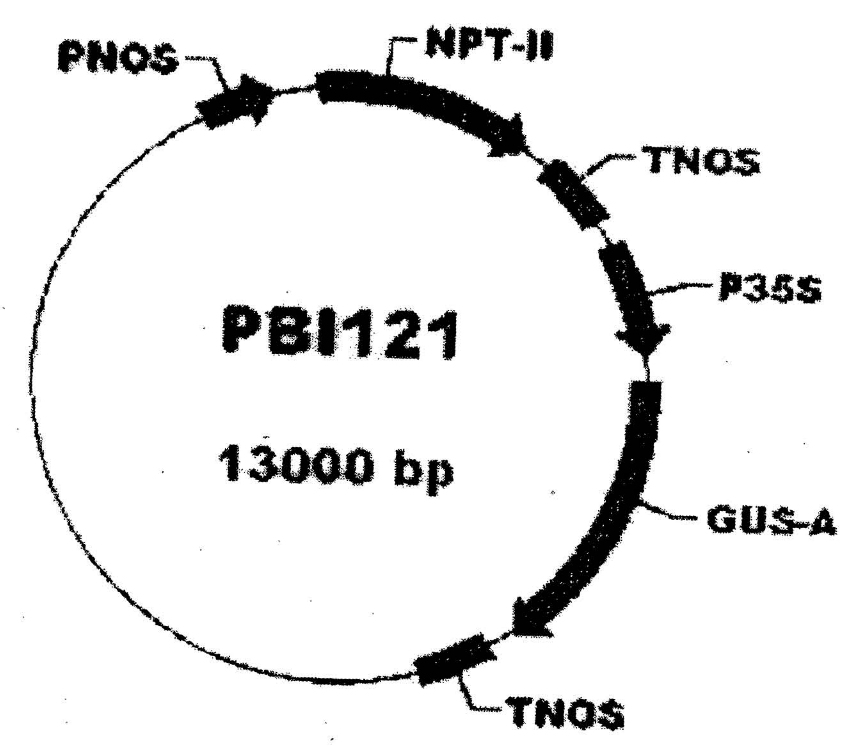
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PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Books

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Limits Preview/Index History Clipboard Details

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  JOURNAL   Plant Mol. Biol. 25 (6), 989-994 (1994)
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  TITLE     A comprehensive set of modular vectors for advanced manipulations
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  REMARK    Full description of constructs
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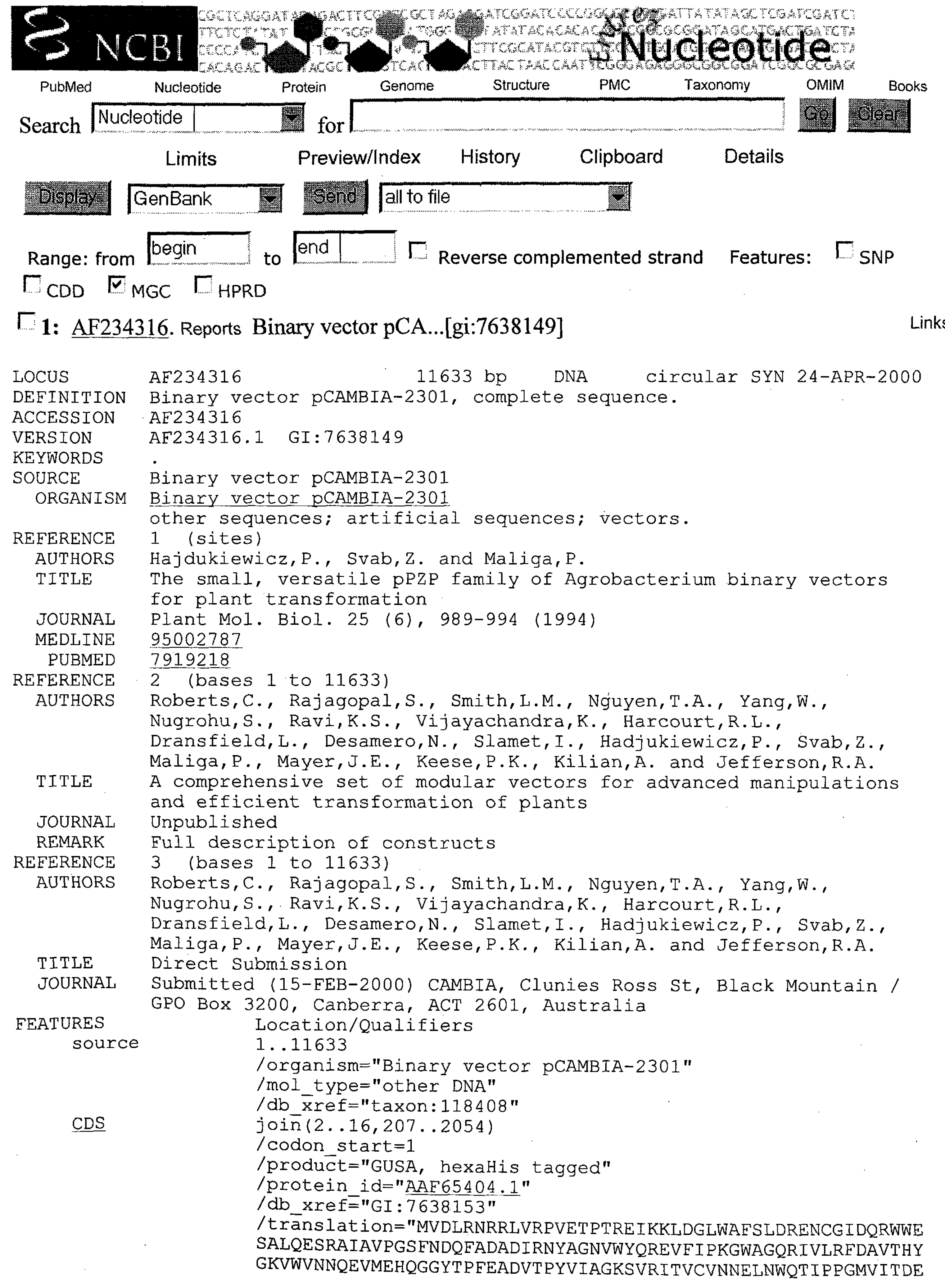

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  JOURNAL   Unpublished
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intron 17..206
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misc_feature 2086..2338
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promoter complement(9796..10576)
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promoter 10724..10778
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11161 gtctcttacg actcaatgac aagaagaaaa tcttcgtcaa catggtggag cacgacacac
```

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11221 ttgtctactc caaaaatatc aaagatacag tctcagaaga ccaaagggca attgagactt
11281 ttcaacaaag ggtaatatcc ggaaacctcc tcggattcca ttgcccagct atctgtcact
11341 ttattgtgaa gatagtggaa aaggaaggtg gctcctacaa atgccatcat tgcgataaag
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11581 cctctatata aggaagttca tttcatttgg agagaacacg ggggactctt gac
//
```

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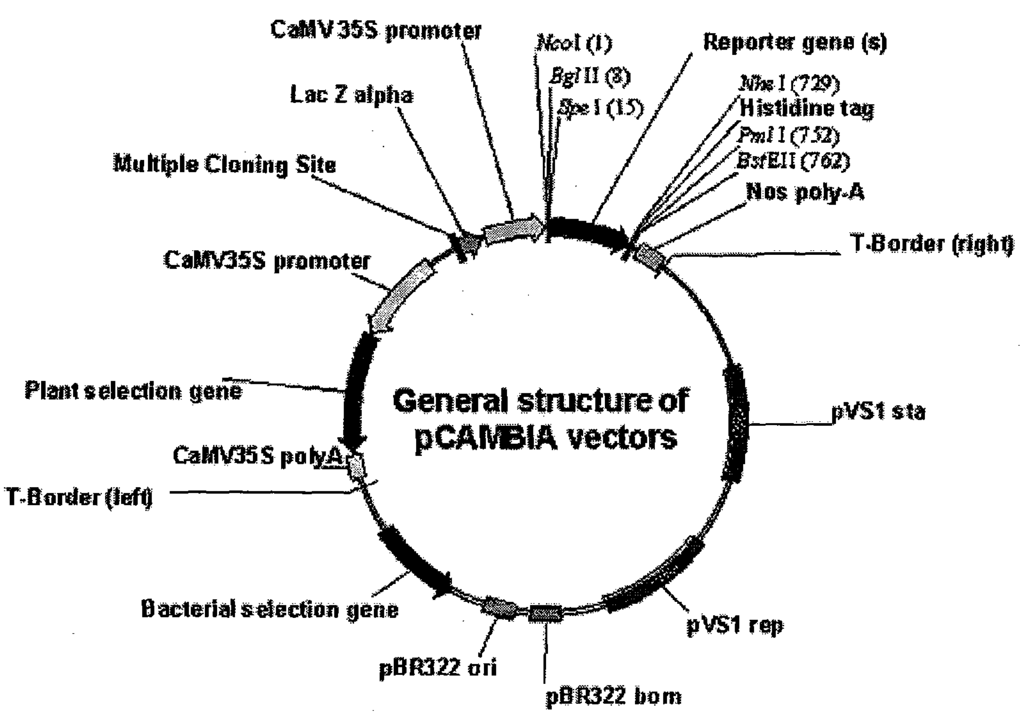
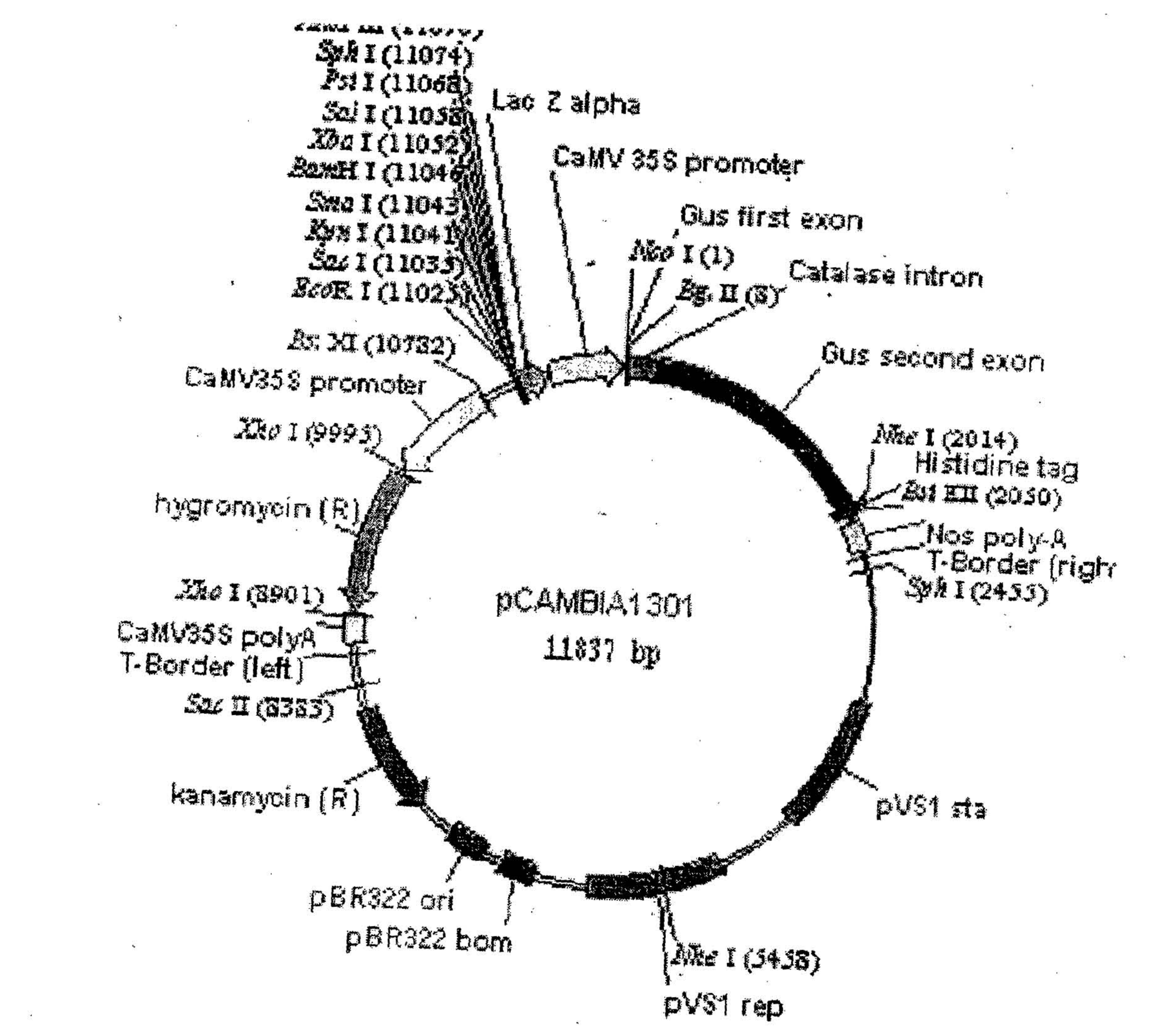

CaMV35S promoter
NcoI (1)
BglII (8)
SpeI (15)
Reporter gene (s)
NheI (729)
Histidine tag
PmlI (752)
BstEII (762)
Nos poly-A
T-Border (right)
Lac Z alpha
Multiple Cloning Site
CaMV35S promoter
Plant selection gene
General structure of
pCAMBIA vectors
pVS1 sta
CaMV35S polyA
T-Border (left)
Bacterial selection gene
pVS1 rep
pBR322 ori
pBR322 bom


Sph I (11074)
Pst I (11068)
Sal I (11058)
Xba I (11052)
BamH I (11046)
Sma I (11043)
Kpn I (11041)
Sac I (11033)
EcoR I (11023)
Lac Z alpha
CaMV 35S promoter
Gus first exon
Nco I (1)
Bgl II (8)
Catalase intron
Gus second exon
Nhe I (2014)
Histidine tag
Bst EII (2050)
Nos poly-A
T-Border (righ
Sph I (2455)
pVS1 sta
Nhe I (5438)
pVS1 rep
pBR322 bom
pBR322 ori
kanamycin (R)
Sac II (8383)
T-Border (left)
CaMV35S polyA
Xho I (8901)
hygromycin (R)
Xho I (9993)
CaMV35S promoter
Bs XI (10732)
pCAMBIA1301
11837 bp