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AGROBACTERIUM TUMEFACIENS MEDIATED GENETIC TRANSFORMATION IN *DENDROBIUM* VARIETY SONIA 17 WITH 1- AMINOCYCLOPROPANE- 1 CARBOXYLIC ACID (ACC) SYNTHASE ANTISENSE GENE

KARTHIKA KARUNAKARAN

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Department of Plant Biotechnology
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522

DECLARATION

I hereby declare that this thesis entitled "***Agrobacterium tumefaciens*** **mediated genetic transformation in** ***Dendrobium*** **variety Sonia 17 with 1- aminocyclopropane-1 carboxylic acid (ACC) synthase antisense gene**" 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 of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Karthika

Vellayani, **KARTHIKA KARUNAKARAN**

23-02-2007. (2004-11-09)

CERTIFICATE

Certified that this thesis "***Agrobacterium tumefaciens*** **mediated genetic transformation in *Dendrobium* variety Sonia 17 with 1- amino cyclopropane-1 carboxylic acid (ACC) synthase antisense gene"** is a record of research work done independently by Ms. Karthika Karunakaran (2004-11-09) 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.

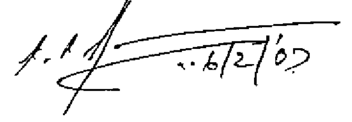
Vellayani,
23-02-2007

Dr. K. RAJMOHAN
Associate Professor and Head,
Department of Plant Biotechnology,
College of Agriculture, Vellayani

APPROVED BY

Chairman:

Dr. K. RAJMOHAN
Associate Professor and Head,
Department of Plant Biotechnology,
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522



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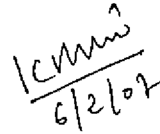
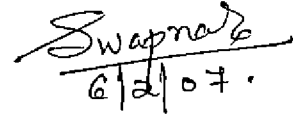
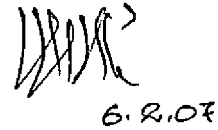
Dr. K. B. SONI
Assistant Professor,
Department of Plant Biotechnology,
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522



Dr. SWAPNA ALEX
Assistant Professor,
Department of Plant Biotechnology,
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522

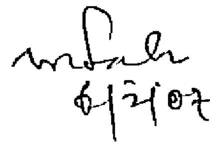


Dr. K. N. ANITH
Associate Professor,
Department of Plant Pathology
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522



EXTERNAL EXAMINER

Dr. T. D. SAHA
Senior Scientist,
Genome Analysis Lab,
Rubber Research Institute,
Kottayam- 686 009



Dedicated to
My Beloved Parents
and Teachers

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LIST OF ABBREVIATIONS

ABA	Absissic acid
ADS	Adenine sulphate
BA	Benzyl adenine
BSA	Bovine Serum Albumin
bp	base pair
2, 4-D	2, 4-dichlorophenoxyacetic acid
DNA	Deoxy ribonucleic acid
dNTPs	deoxy Nucleotide Tri Phosphates
EDTA	Ethylene Diamine Tetraacetic Acid
GA	Gibberellic acid
gus	β-D-Glucuronidase
kb	kilo base pair
Kn	Kinetin
M	Molar
μM	Micromolar
MS medium	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
npt	Neomycin phosphotransferase
OD	Optical density
PCR	Polymerase Chain Reaction
pH	Per Hydrogen
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride sodium citrate
TAE	Tris acetic acid EDTA
Tris Cl	Tris (hydroxy methyl) amino methanehydrochloride
vir	Virulence genes
YEM	Yeast Extract Mannitol

Introduction

1. INTRODUCTION

Cut flowers are important foreign exchange earners. The world cut flower market is growing at an annual rate of 6-9 per cent. The global consumption of cut flowers is valued at 55 billion US dollars. Orchids are among the important cut flower crops. They are known for their bewitching and beautiful flowers with variety of patterns.

Orchid industry is grossly influenced by changes in preferences and aesthetic values. Novel varieties with distinct traits are always welcome. New varieties remain at the prime position only for a short period. Hence there is an ever increasing demand for new varieties. At the international level the cut flower industry is supported by efficient breeding programmes. Unfortunately in India not much centers are involved in orchid breeding. Hence the orchid industry in India has to depend upon international varieties. The new varieties reach India only after they are off the prime position. This makes it difficult for the orchid industry to compete with the international market. Traditional breeding has its own limitations as it is time consuming and laborious. Genetic engineering offers an option for the incorporation of desirable genetic traits in a faster and more precise manner complimenting traditional breeding. Genetic transformation is being effectively used for the rapid improvement of floricultural crops world over.

Among the different epiphytic orchids grown in India, *Dendrobium* has prime position owing to attractive blooms which are in high demand in domestic and international markets. *Dendrobium* hybrids are the most suitable and popular among the commercial orchids grown in Kerala. Sonia (*D. caesae* x *D. Tomie Drake*) is well known for its attractive flowers. They have reasonably good vase life. However, further improvement of the vase life can reduce the per day cost of the flower.

Post harvest longevity of flowers is critical in the cut flower industry. Ethylene has been reported as the signal that initiates and coordinates senescence in plants. The genetic manipulation of ethylene biosynthetic and signal transduction pathway through antisense RNA technology has resulted in flowers with delayed senescence as in the case of carnation, petunia, and chrysanthemum. Manipulation of the genes involved in senescence signaling pathway is the best genetic target to delay senescence without affecting other fundamental events. The enzyme 1- amino cyclopropane 1- carboxylic acid (ACC) synthase is the core enzyme involved in the synthesis of the gaseous plant hormone, ethylene. ACC synthase converts S- adenosyl methionine (SAM) to ACC which in turn is converted to ethylene by ACC oxidase. By suppressing the expression of ACC synthase using antisense gene, the ethylene production can be reduced.

There are only a few reports on *Agrobacterium* mediated genetic transformation in orchids. Till date, no report has been published in India regarding genetic transformation of orchids. The *Agrobacterium* mediated genetic transformation using *gus* gene in orchids has been standardized in the Department of Plant Biotechnology, College of Agriculture, Vellayani (Swarnapiria. 2004). The introduction of the ACC synthase antisense gene in *Dendrobium* Sonia 17, using the leads of this work would be useful in improving the vase life of the flower.

In view of the above facts, present investigation was conducted with the following objective:

Genetic transformation of *Dendrobium variety* Sonia 17 with 1-aminocyclopropane-1 carboxylic acid (ACC) synthase antisense gene for improving the vase life of flowers.

Review of Literature

2. REVIEW OF LITERATURE

The study "*Agrobacterium tumefaciens* mediated genetic transformation in *Dendrobium* Sonia 17 with 1- aminocyclopropane1- carboxylic acid (ACC) synthase antisense gene" aims at standardization of protocol for transforming orchid for improved vase life. The review of literature related to the above work is presented in this chapter.

2. 1 THE ORNAMENTAL VALUE OF ORCHID

Orchids are very distinctive ornamentals. They occupy 13 per cent of the total land area devoted to cut flower (Singh, 2005). Orchid growing is a multi-million industry, most notably in the cut flower trade (Khairol and Auni, 1991). *Dendrobiums* are the popular commercial orchid grown in Kerala. Approximately 24.3 million stalks of orchid cut-flowers were produced in 2000 with *Dendrobium* topping the list at 13.1 million stalks. They are mass-propagated by the floral industry and widely commercialized for their decorative value.

The vase life is one of the characteristics determining the commercial value of ornamentals (Reid and Wu, 1992). Ethylene is a primary plant hormone involved in the senescence of cut flowers (Borochov and Woodson, 1989). A large amount of ethylene is produced, mostly in the petals, several days after full opening of the flower (Woodson *et al.*, 1992). The increased ethylene production promotes the in-rolling of petals resulting in wilting of the flower. The time of onset of ethylene production and the amount of ethylene produced in the flowers vary with the cultivar, and thus influence their vase life (Nukui *et al.*, 2004).

Bewan *et al.* (1983) first reported plant genetic transformation using *Agrobacterium tumefaciens* in tobacco. Since then gene delivery system has been widely used for the transfer of desirable genes. The identification and availability of genes of horticultural interest are increasing. There is increasing consumer

interest in florist products world wide. This signals that tremendous economic benefits may be gained by the creation of new and improved orchid hybrids.

For molecular breeding to be feasible the tissue to be genetically engineered must give rise to plants. To achieve this, a suitable protocol for mass production of protocorm like bodies (PLBs) in orchid *Dendrobium* Sonia 17 has been standardised. Many economically important hybrids developed PLBs slowly in culture (Arditti and Ernst, 1993). In recent years *in vitro* techniques for micropropagation and flowering of orchids have opened new avenues of research into the flowering process (Goh, 1996).

2. 2 *IN VITRO* PRODUCTION OF PROTOCORM LIKE BODIES (PLBs)

2. 2. 1 Establishment of Protocorm Like Bodies

Sagawa and Shoji (1967) reported that Protocorm like bodies (PLBs) are obtained from the culture of shoot apices *in vitro*. Stewart and Button (1975) opinioned that callus could be differentiated from shoot apex. The callus further proliferated by the regeneration of PLBs (Jonojit and Nirmalya, 2003).

2. 2. 2 Sterilisation Techniques

Jordan (1965) reported the use of chemical treatments for the sterilisation of mature orchid seeds. Mitra (1971) used chlorine water for the sterilization of explants. Rosa and Laneri (1997) used 70 per cent ethanol for sterilising pods. Saiprasad *et al.* (2002) reported the transfer of explants to sterile water in conical flask and surface sterilized with 0.1 per cent mercuric chloride and two drops of tween 20 for 180 sec. The explants were then washed with sterile water to remove the sterilants from the explants.

2. 2. 3 Effects of Organic Additives on the Development of PLBs

Intuwong and Sagawa (1973) reported that organic additives like coconut water induced cell division in non dividing cells and promoted morphogenesis and mass multiplication of protocorm in orchids. Kim *et al.* (1970) found that the optimum concentration of coconut water in the medium was 10 to15 per cent. Morel (1974) enumerated the effect of organic additives like coconut water in rapid protocorm multiplication in orchids. Kusumoto (1979) reported that yeast extract accelerated the production of protocorms in *Cymbidium.* Soediono (1988) found that VW medium supplemented with 15 per cent coconut water and 10 per cent NAA led to rapid protocorm proliferation and enhanced seedling growth in *Dendrobium* Jaquelyn Thomas. Devi *et al.* (1990) reported that the addition of pine apple juice to VW medium enhanced germination and accelerated the leaf and root growth. Agarwal *et al.* (1992) found that casein hydrolysate supported the rapid proliferation of multiple shoots from stem node segments. Sharon *et al.* (1992) reported the use of basal medium with 15 per cent coconut water for raising the protocorm of *Dendrobium* Snowfire from immature seeds. Lekharani (2002) pointed out that coconut water 200 ml l^{-1} was the best for early protocorm differentiation and rapid seedling growth. Swarnapiria (2004) enumerated that coconut water 150 ml l^{-1} was suitable for the *in vitro* proliferation of PLBs.

2. 2. 4 Effect of Charcoal on the Development of PLBs

Frigborg *et al.* (1978) attributed the beneficial effects of activated charcoal in the adsorption of inhibitory compounds like phenols produced by the tissues in culture. According to Hinnen *et al.* (1989) charcoal enhanced the growth and development of orchid seedlings. Swarnapiria (2004) reported that activated charcoal 1.5 g l^{-1} is suitable for the *in vitro* proliferation of PLBs

2. 2. 5 Effect of Carbon Source on the Development of PLBs

Orchids require an external supply of carbohydrates for their growth and differentiation. Arditti *et al.* (1992) reported that orchid PLBs have the ability to utilize various carbohydrates. Hew *et al.* (1988) reported that when apical meristems were cultured in VW medium, fructose was more readily utilized than other sugars. Honjo *et al.* (1988) observed that increase in fresh weight was markedly affected by sucrose. Paek and Yeung (1991) reported that sucrose was found to be the most effective in shoot induction.

2. 2. 6 Effects of Culture Media and Plant Growth Substances on the Development of PLBs

Orchids require auxins and/or cytokinins for the formation of PLBs and plantlet development (Adritti and Ernst 1993). Mitra *et al.* (1976) revealed that triacontanol (TRIA) can be used as an effective growth regulator in the micropropagation of *D. nobile.* The formation of PLBs depends on auxin cytokinin ratio of 0.12 for *Phalaenopsis* leaf culture (Tanaka and Sakanishi, 1985) and 0.42 for *Dendrobium antennatum* Lindley (Kukulczanka and Wojiechowska, 1983).

Liu and Zhang (1998) reported that B5 and half strength MS, added with 10 per cent of banana aqueous extract and 2 mg^{-1} NAA could be used as the best plantlet strengthening medium for *Dendrobium candidum.* Pyati *et al.* (2002) obtained the *in vitro* propagation of *Dendrobium macrostachyum*, cultured on Murashige and Skoog (MS) basal medium and MS medium supplemented with N6-benzyladenine (2.22, 4.44 and 8.88 mM), kinetin (2.32, 4.65, and 9.29 mM) and coconut water (5, 10 and 15 per cent) individually or in combination with 2.69 mM alpha- naphthalene acetic acid (NAA).

Chen *et al.* (2000) reported the generation of compact and yellow-white embryogenic calli formed from root tips and cut ends of stem and leaf segments of *Oncidium* on half strenght MS basal medium supplemented with 1- phenyl- 3 (1, 2, 3- thiadiazol- 5)- urea (TDZ, 0.1-3 mg l^{-1}), 2, 4-dichlorophenoxyacetic acid (2,4-D, 3-10 mg l^{-1}) and peptone (1 g l^{-1}) for 4 to 7 weeks.

Saiprasad *et al.* (2002) obtained several clumps of PLBs on culturing the shoot-tips of *Dendrobium* sonia on MS medium with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA for 120 days. Jiang and Zheng (2003) reported that half strenght MS + 0.1 mg^{-1} NAA was suitable for the propagation of PLBs. Luo *et al.* (2003) reported the induction of PLBs of *D. huoshanenese* cultured on basal MS medium.

Chang *et al.* (2004) reported half strenght MS + NAA 0.5 mg l^{-1} could be used as the optimal protocorm multiplying media for *Dendrobium.* Tang *et al.* (2005) supplemented half strength MS medium with 1.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA and found very beneficial to the protocorm differentiation and propagation of *D. candidum.* The highest root number and length was observed in plants growing in half strength MS medium containing 0.5 mg l^{-1} NAA. Hou and Guo (2005) found that diluted MS without any growth regulators promotes the growth of protocorms but inhibit differentiation.

2. 3 IMPROVEMENT OF ORNAMENTALS BY GENETIC MANIPULATION

Genetic engineering has been widely used for the improvement of ornamentals for the incorporation of desirable traits. Dolgov *et al.* (1997) reported an efficient regeneration protocol for successful *Chrysanthemum* transformation. Transgenic plants with *bt* toxin, *rolC, chs* and AFP genes were produced. De Jong *et al.* (2000) established procedure for the genetic modification in *Chrysanthemum* by introducing the *CryI C* gene to create plants resistant to insects. Mitiouchkina and Dolgov (2000) transferred the *rol C* gene sequence under 35S promoter on plasmid pPCV002 in *Agrobacterium* strain GV3101 in *Chrysanthemum.* The transformed lines demonstrated plants with

wider petals and changed forms, dramatic changes in phytohormones content especially, in cytokinin auxin ratio were observed.

Mitiouchkina *et al.* (2000) transferred the full-length cDNA copy of *chs* gene from *A. majus* in *Chrysanthemum* genome of Parliament variety in antisense orientation. The transgenic lines demonstrated suppression of flower colour compared to control plants. Wang *et al.* (2004) reported transformation of snowdrop lectin gene for aphid resistance in *Chrysanthemum.* Narumi *et al.* (2005) reported transformation of *Chrysanthemum* with mutated ethylene receptor genes mDG-ERS1, for reduced ethylene sensitivity.

Deroles *et al.* (2000) modified the chalcone biosynthesis in *Petunia hybrida* by introducing a *Chs* cDNA from *Medicago sativa* under the control of the 35S CaMV promoter into the white flowered Mitchell line of *Petunia.* The flower color was changed from white to pale yellow. Esposito *et al.* (2000) reported the introduction of gene *ech-42*, encoding an endochitinase from the antagonist fungus *Trichoderma harzianum*, alone or in combination with the osmotin gene from *Nicotiana tabacum* into a *Petunia hybrida* pure line by the *Agrobacterium tumefaciens* leaf disc system. The transgenic lines revealed a statistically significant reduction of the disease symptoms in comparison with the controls.

Miroshnichenko and Dolgov (2000) reported *Agrobacterium* mediated transformation procedure for carnation using the hygromycin resistance gene (*Hpt*) as a selective agent. Ovadis *et al.* (2000) used a highly efficient and reliable transformation procedure for carnations. Carnations with colour modifications and altered plant morphology and performance were obtained by introducing an *fht* antisense gene and a *rol C* gene, respectively.

Babu and Chawla (2000) reported *Agrobacterium* mediated genetic transformation in gladioli using gus gene. Loffler *et al.* (2000) reported the genetic transformation of gladioli for fusarium resistance using particle bombardment with gold particles coated with a construct harboring the *gus*

reporter gene and the *pat* selection gene. Suzuki *et al.* (2001) reported *Agrobacterium* mediated transformation in lillaceous ornamental plants

Condliffe *et al.* (2001) reported the *Agrobacterium* mediated genetic transformation in rose cultivars.

Ketsa *et al.* (2004) studied the role of ethylene in ovary growth of *Dendrobium* 'Pompadour'. Ethylene synthesis was inhibited using aminooxyacetic acid (AOA). AOA delayed the time to wilting of 50 per cent of the flowers. Chan *et al.* (2005) reported gene stacking on *Phalaenopsis* orchid by double transformation to enhance the resistance of orchids to both viral and bacterial phytopathogens. This is the first report describing a transgenic *Phalaenopsis* orchid with dual resistance to phytopathogens.

2. 4 TRANSFORMATION USING ACC SYNTHASE ANTISENCE GENE

Altvorst *et al.* (1994) reported that the senescence of flower petals is a highly regulated developmental process which requires active gene expression and protein synthesis. The gaseous phytohormone ethylene plays a critical role in the regulation and coordination of senescence processes. The ethylene biosynthesis path way could be regulated through antisense RNA technology and ethylene response could be blocked. This could improve post harvest longevity of cut flowers like carnation.

Iwazaki *et al.* (2004) reported *Agrobacterium* mediated gene transformation of Carnation (*Dianthus caryophyllus* L. cv. Nora) plants using ACC synthase antisense (*DC-ACS1*) gene. The transgenes showed suppressed ethylene production during natural senescence as compared with flowers of the non-transformed control. Shaw *et al.* (2004) reported *Agrobacterium* mediated genetic transformation in *Petunia hybrida* using *boers*, an ethylene receptor sensor gene of *Brassica oleracea.* Transformed plants produced apparently larger flowers.

Theologis *et al.* (1993) produced transgenic tomato plants expressing antisense RNA of the key enzyme in the ethylene biosynthetic pathway, 1-amino cyclopropane-1-carboxylate (ACC) synthase using the constitutive CaMV 35S and fruit specific E8 promoters. Fruits expressing antisense LE-ACS2 RNA produce less ethylene and failed to ripen. Ju *et al.* (1994) inserted the *PG* gene for studying the inhibition effects of its antisense RNA on the expression of *PG* gene in transgenic tomato plants. The results suggested that the expression of the anti-*PG* gene effectively inhibited the expression of endogenous PG gene.

Nakatsuka *et al.* (1997) transformed tomato with ACC synthase antisense gene and observed strong positive feed back in ACC synthase and ACC oxidase gene transcriptional level in tomato fruit, even at the stage with a burst of ethylene production. Liu *et al.* (1998) reported *A. tumefaciens* mediated transformation of tomato using ACC synthase antisense gene. The amount of ethylene released from transgenic tomato fruits was reduced significantly (30%) of that released by non-transformed controls. The shelf life of transgenic tomato fruits was at least 60 days at room temperature without significant change in hardness and color. Xiong *et al.* (2003) reported the construction of binary plant expression vector pOSACC in which the double- antisense ACC oxidase and ACC synthase fusion gene was introduced, controlled by fruit-specific 2A11 promoter. The transgenic tomatoes showed the characteristics of prolonged shelf life over 50 days. Ethylene released from the transgenic fruits was reduced significantly to about 9.5 per cent of that released by non-transformed controls.

Laurena *et al.* (2000) prevented the post-harvest loss in papaya by down regulating ethylene biosynthesis through antisense expression of ACC synthase gene. ACS_2, a good candidate gene of ACC synthase antisense strategies, was used to regulate fruit ripening. The gene was inserted in an antisense orientation in two types of vector constructs for papaya transformation.

2. 5 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION IN ORCHID

There is substantial interest in the genetic improvement of orchid. Orchids form the largest family of flowering plants with 25, 000 species that are commercially grown (Arditti, 1992). Kuehnle and Sugii (1992) first reported the genetic transformation in orchids. Chia *et al.* (1994) reported the genetic transformation in orchid using firefly luciferase gene. Knapp *et al.* (2000) reported the transformation of *Dendrobium* using *bar* gene. Yu *et al.* (2001) reported the generation of transgenic orchid (*Dendrobium* Madame Thong-In) plants by inoculating thin section explants from PLBs with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector that carried the orchid *DOH1* antisense gene.

Liau and Lu (2003) reported for the first time the successful transformation into PLBs of *Oncidium* orchid using *Agrobacterium tumefaciens* strain EHA105 harbouring the sweet pepper ferredoxin-like protein (pflp) which conferred resistance against soft rot disease. Liau *et al.* (2003) established a transformation procedure via *Agrobacterium tumefaciens* for an important *Oncidium* orchid cultivar. An expression vector containing *hptII* and *gusA* genes driven by the cauliflower mosaic virus (CaMV) 35S promoter was successfully introduced into the PLBs. You *et al.* (2003) successfully transformed protocorm-like bodies of *Oncidium* orchid using *Agrobacterium tumefaciens*. The pflp gene was used as selection marker and *Erwinia carotovora* as the selection agent, thereby obtaining transgenic plants without the use of an antibiotic selection agent.

Belarmino *et al.* (2000) reported *Agrobacterium* mediated genetic transformation of *Phalaenopsis* orchid for hygromycin resistance. Men *et al.* (2004) reported the transformation in *D. nobile* by biolistic bombardment. The plasmid pCAMBIA1301 encoding beta-glucuronidase gene (*gus-int*) and a hygromycin phosphotransferase (*hpt*) gene were introduced into the PLBs. Mishiba and Chin (2005) reported *Agrobacterium* mediated transformation of

Phalaenopsis by targeting protocorms at an early stage after germination. Chan *et al.* (2005) reported the gene stacking on *Phalaenopsis* orchid for resistance against both viral and bacterial phytopathogens. Gene stacking was applied by double transformation. This enabled the expression of dual (viral and bacterial) disease resistant traits.

2. 5. 1. Factors Affecting *Agrobacterium* Mediated Gene Transfer

2. 5. 1. 1 Explants for Genetic Transformation

For molecular breeding to be feasible, the tissue to be genetically engineered must give rise to plants. The gene transfer method and the gene expression vectors used must be compatible with the plant genotype and the tissue to be treated.

Chia *et al.* (1994) developed bioluminescent orchid from the PLBs of *Dendrobium* White Angel. Yu *et al.* (2001) reported the production of transgenic orchids (*Dendrobium* Madame) by the co cultivation of thin section explants from PLBs with *A. tumefaciens.* Tee *et al.* (2002) reported the genetic transformation of *Dendrobium* sonia 17 using three different morphological callus types of *in vitro* inflorescence as target tissues. Chia *et al.* (2002) obtained transgenic orchids for beta- glucuronidase and hygromycin resistance by cocultivation of PLBs with *Agrobacterium tumefaciens.* Men *et al.* (2004) reported genetically transformation of *Dendrobium nobile* by particle bombardment using PLBs as target explants.

Chan *et al.* (2003) obtained the regeneration of *Cymbidium niveomarginatum* after co- cultivation of their rhizome sections with *A. tumefaciens.* Liau *et al.* (2003) used PLBs of *Oncidium* as explants for *Agrobacterium* mediated genetic transformation.

Anzai *et al.* (1995) reported the use of outgrowths of leaf segments of *Phalaenopsis* resembling PLBs as explants to generate stable transformants.

Hsieh *et al.* (1997) reported the transformation of PLBs of *Phalaenopsis* varieties with *Agrobacterium tumefaciens*. Belarmino and Mii (2000) obtained genetically transformed plants of *Phalaenopsis* orchid after co-cultivation of PLBs with *A. tumefaciens.* Mishiba *et al.* (2005) recommended *Agrobacterium* mediated transformation of *Phalaenopsis* targeting protocorms at an early stage after germination. Chan *et al.* (2005) gave the first report describing a transgenic *Phalaenopsis* orchid with dual resistance to phytopathogens where they used PLBs as the explants.

2. 5. 1. 2. Agrobacterium Infectivity

Agrobacterium tumefaciens is a soil-borne bacterial pathogen of the family Rhizobiaceae which normally infects wounds of dicot plants at the root-shoot interface and causes severe damages due to the crown gall disease (Nesme *et al.*, 1987). These phytopathogenic bacteria use genetic engineering processes to subvert the host plant cell metabolic machinery. Hawes *et al.* (1989) reported that the parasitized cells are also induced to proliferate and the resulting crown gall tumour disease is a direct result of the incorporation of a region of transfer DNA, T-DNA, from a large (150-250 kb) circular Ti (tumour inducing) plasmid, carried by *A. tumefaciens*, into the host plant genome. Hooykaas and Shilperoort (1992) reported that the natural transformation process, together with any foreign DNA placed between the T-DNA borders sequences could be transferred to plant cells. This could be used for construction of a vector and bacterial strain systems for plant transformation.

The advent of recombinant DNA technology has enabled the use of *A. tumefaciens* for genetic transformation, despite the fact that there are many other efficient methods for direct gene transfer (Christou, 1996). When suitably modified, *Agrobacterium* has become the most effective vector for gene transfer in plants (Gelvin, 2003). *Agrobacterium* mediated transformation is the preferred method for its simplicity, cost effectivity, little re-arrangement of transgenes,

ability to transfer relatively longer DNA segments (Hamilton *et al.,* 1996) and preferential integration of foreign genes into transcriptionally active regions (Ingelbrecht *et al.,* 1991). This ensured proper expression of transgenes in transgenic plants (Hernandez *et al.,* 1999). The transformation has been reported to be depended on the variety, types of explants, delivery system, the strain, and conditions of co- cultivation, selection method, and mode of regeneration (Mathis and Hinchee, 1994). Nitish *et al.* 2004 indicated that the leaves with glabrous surface having lower *q* (larger surface area covered by water droplet), higher phenol and wax content were more suitable for *Agrobacterium* infection. Baron *et al.* (2001) reported that elevated temperature differently affected virulence.

Agrobacterium mediated genetic 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 process of genetic transformation in monocotyledons (Smith and Hood, 1995). Infection of plants or explants by *Agrobacterium* is basically a host-pathogen reaction (DeCleene and DeLey, 1976). Biao *et al.* (1998) reported that explants with high levels of bacteriostatic polyphenols are recalcitrant to *Agrobacterium* infection. *Agrobacterium* mediated gene transfer is usually generalized to produce simpler integration patterns, less rearrangements within inserts and reduced problems with cosupression and instability over generations, compared to methods based on direct gene transfer (Komari and Kudo, 1999) with higher efficiency of stable transformation with many single copy insertions (Veluthambi *et al.,* 2003).

2. 5. 1. 3. Strain specificity

The limited host range specificity of *Agrobacterium* is a well documented fact (Hawes *et al.,* 1989). There are differences in the susceptibility of *Agrobacterium* infection between species and even between cultivars and

genotypes of the species. Hence the best method is to transform with different strains harbouring a good selectable marker, till we get the genotype/strain combination. Several *Agrobacterium tumefaciens* strains varying in chromosomal background, *vir* helper plasmid and binary vector plasmid should be tested for their competence to transform. Hawes *et al.* (1989) reported that even within a particular species, the range of host specificity varies widely amongst the different cultivars or genotypes.

Agrobacterium tumefaciens strain GV3101 containing the plasmid vector pMP90 containing the marker gene for kanamycin resistance and a reporter gene for *gus*, both controlled by the pNOS promoter derived from nopaline synthase gene was used to transform *Atropa belladonna* plants (Negoianu *et al.*, 2002). Le Flem-Bonhomme *et al.* (2004) used *Agrobacterial* strain GV3101 to transform *Papaver somniferum* var. album for the hairy root induction. Leal *et al.* (2004) reported *Agrobacterium tumefaciens* mediated transformation of *Paracoccidioides brasiliensis* using the strain GV3101 carrying the vector pAD1625. Song and Sink (2004) reported *Agrobacterium tumefaciens*-mediated transformation of blueberry (*Vaccinium corymbosum* L.) using GV3101.

Zhao *et al.* (2005) reported the genetic transformation of *Festuca arundinacea* Schreb with GV3101. Cai *et al.* (2006) established that the *Agrobacterium* strain used for agro-inoculation significantly affects the VIGS efficiency. Strain GV3101 was highly effective for the development of a Virus-Induced Gene-Silencing System for functional analysis of the RPS2-dependent resistance signalling pathways in Arabidopsis. Oosumi *et al.* (2006) reported high-efficiency transformation of the diploid strawberry (*Fragaria vesca*) by *Agrobacterium* strain GV3101.

2. 5. 1. 4. Bacterial density

Concentration of bacterial cells in the induction medium is an 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 explants. Some species are very sensitive to bacterial infection and hence very low density of bacterial population is used. So optimum density for each species of *Agrobacterium* is essential for efficient transformation.

Hoekema *et al.* (1983) reported that *Agrobacterium tumefaciens* was grown for two days in YEP medium containing appropriate antibiotics at 28°C until an OD_{600} of 1.0 was obtained. Lichtenstein and Draper (1986) attempted transformation using a bacterial suspension with a final OD value adjusted to 0.6-0.8.

Belarmino and Mii (2000) used a bacterial suspension of *A. tumefaciens* at 1:10 (vol/ vol) for the transformation of *Phalaenopsis.* Babu and Chawla (2000) reported the use of *Agrobacterial* suspension; the optical density of the culture was measured at 600 nm and adjusted to 0.1 by dilution or cultivation. Mishiba *et al.* (2000) used the bacterial suspension diluted to one- tenth concentration with the liquid callus induction medium for the genetic transformation of lavender. Suzuki *et al.* (2001) transformed the embryonic callus of *Agapanthus* using the bacterial suspension diluted to a final concentration of 0.2. Seo *et al.* (2003) reported that in *Chrysanthemum* cv Puma, the explants were cocultivated with a bacterial suspension diluted in the ratio of 1:50. Park (2005) reported the use of *Agrobacterium* grown to log phase in YEP liquid medium (OD_{600} 0.7 – 0.9) for infection.

2. 5. 1. 5. Co- cultivation

For transformation to be efficient, the induced *Agrobacterium* should have access to cells that are competent for transformation. The explants used for transformation was exposed to the bacterial culture media in the induction with optimum cell density. Both the composition of the media and the time of induction decided the efficiency of transformation. MS medium was used for the induction, segregation and culturing of bacteria. For inoculation, the explants were immersed in induction medium for a specific period of time, which depended on bacterial population, type of vector and explants used for transformation.

Hsieh *et al.* (1997) reported a co-cultivation method for transforming *Phalaenopsis* varieties *in vitro* with *A. tumefaciens* strain EHA 105. PLBs at the ten day proliferation stage were the optimal material for infection. Scanning electron microscopy revealed that *Agrobacterium* attached very well to the surface of the PLBs. Murshige and skoog salts and vitamins were used for the preparation of induction medium (Nagaraju *et al.*, 1998) for the transformation of *Gerbera hybrida.*

A co-cultivation period of two days using 1:10 dilution resulted in transformed plants (Mishiba *et al.*, 2000). The efficiency of transformation was markedly increased by co-cultivation of cell clumps with *A. tumefaciens* for ten hours (Belarmino and Mii, 2000). Yu *et al.* (2001) reported *Agrobacterium* mediated genetic transformation of *Dendrobium* with the class1 knox gene DOHI The transformation was performed through two consecutive stages of co-cultivation, with the first stage occurring on antibiotic free medium for three days and subsequent stage on medium containing 50 mg-1 carbenicillin for 3- 4 weeks.

In *Chrysanthemum*, Kudo *et al.* (2002) reported that four days of co-cultivation at 24°C was the optimum using EHA 101 and PIG121Hm plasmid. Park *et al.* (2005) reported transformation methodology using explants cultured on MS media with growth regulators and three days of co-cultivation with the *Agrobacterium* on this same medium. Chan *et al.* (2003) reported maximum GUS expression when the PLBs were sonicated for 150 seconds and co-cultivated for three days at 26°C.

2. 5. 1. 6. Use of acetosyringone

The Ti plasmid virulence (*vir*) loci encode functions essential for the transfer of the T-DNA element from *Agrobacterium tumefaciens* to plant cells. These *vir* genes and thus the virulence of *Agrobacterium* are stimulated by certain phenolic compounds such as acetosyringone and hydroxyacetosyringone secreted from the wounded plant tissue. When *A. tumefaciens* get attached to a plant cell, and the *vir* genes are induced, which help the transferring of the T- DNA to the plant cell. Thus these compounds such as acetosyringone greatly enhance transformation and are now routinely added to transformation experiments.

Babu and Chawla (2000) resuspended the bacterial pellet in Murshige and Skoog medium supplemented with 18.6 mM kinetin supplemented with 100 µM acetosyringone. Mishiba *et al.* (2000) reported that the transformation efficiency of lavender was improved by the presence of acetosyringone (100µM) in co-cultivation medium. The effectiveness of acetosyringone might be assumed due to the *vir* G gene harboured by the vector, activated by the presence of acetosyringone. Belarmino and Mii (2000) reported that the efficiency of transformation in *Phalaenopsis* was markedly increased by the ten hour co-cultivation of cell clumps with *A. tumefaciens* that had been induced with 200µM acetosyringone and by the inclusion of 50 µM acetosyringone in the co-cultivation medium.

Chan *et al.* (2003) soaked the PLBs in bacterial inoculum for one hour and then transferred them to a medium with 5.0 per cent glucose and 100μM acetosyringone for the co-cultivation for three days to obtain transformants in *Phalaenopsis*. Mishiba *et al.* (2005) subcultured the protocorms on acetosyringone containing medium two days before *Agrobacterium* inoculation gave the highest transformation efficiencies.

2. 5. 1. 7. ***Elimination of bacteria after co-cultivation***

Complete elimination of bacteria from the explant after co-cultivation is very essential. Over growth 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 should be such that it kills the bacteria without affecting the growth and organogenesis of the explants. The most commonly used antibiotic for this purpose is cefotaxime. Belarmino and Mii (2000) used 300 mg l^{-1} cefotaxime to eliminate the bacteria after co-cultivation of the *Phalaenopsis* cell clumps with *A. tumefaciens.* These antibiotics can suppress *Agrobacterium* growth very effectively and promote the regeneration of transformed cells into mature plantlets. Babu and Chawla (2000) used 100 mg l^{-1} cefotaxime after co-cultivation and incubated the explants for three days to arrest *Agrobacterium* growth. Mishiba *et al.* (2000) reported the use of 200 mgl^{-1} cefotaxime for the elimination of bacteria.

2. 5. 1. 8. ***Marker and reporter genes***

Selection of transformed cells was a key factor in developing a successful genetic transformation system (Chia *et al.*, 1994). Single dominant gene encoding suitable resistance to a selective agent was used as a marker. The reporter genes were used to analyze the function of promoters and other gene regulatory sequences. These genes did not disrupt the plant regeneration, but allowed the selection of transformed cells. Transformation vectors were constructed with a

reporter gene or a selectable marker, which indicated successful incorporation and expression of the introduced genes. The reporter gene and selectable marker gene were fused to the same plant promoter that was fused to the foreign gene of interest.

The most widely used antibiotic marker is kanancycin, used in the first transformation experiments (Fraley *et al.,* 1983). Neomycin phosphotransferase II (*nptII*) gene from transposon Tn5, detoxify neomycin, kanamycin and G418 by phosphorylation. An *et al.* (1986) rported its use in dicotyledon system including tobacco, potato and tomato Hygromycin phosphotransferase (*hptIV*) governs resistance to hygromycin. This gene isolated from *E coli* has been placed under various promoters and has been successfully used in strawberry (Nehra *et al.,* 1990).

Herbicide markers function in the same way as antibiotic markers. The *bar* gene isolated from *Streptomyces hygroscopicus,* confers resistance to the herbicide phosphinothricin, the active ingredient of Bilaphos and Basta. The *'bar'* gene codes for phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin, an irreversible inhibitor of glutamine synthase. This gene has been inserted and expressed in rape (De Block *et al.,* 1989) and alfalfa (Krieg *et al.,* 1990).

An alternative to antibiotic selection is the use of the firefly luciferase gene. Chia *et al.* (1994) reported the use of a marker for the transformation of the orchid *Dendrobium,* thus producing the first fluorescent orchid. The product of this gene produced light upon reaction with luciferin, which could be detected with a camera photomultiplier. Another reporter gene commonly used in the transformation is anthocyanin. Upon activation, this reporter system produces a reddish purple pigment in transformed tissue (Ludwig *et al.,* 1990). The green fluorescent protein (GFP) is efficiently expressed in plant cells and it is used as a slelectable marker (Tee *et al.,* 2002) in *Dendrobium* sonia 17.

2. 5. 1. 9. Selection of transformed cell

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-transformant 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 per cent sucrose and 50- 100 mg l-1 kanamycin sulphate. Nan and Kuehle (1995) used 100- 200 mgl-1 kanamycin for the selection of transformed *Dendrobium.* Hsieh *et al.* (1997) obtained transformants of *Phalaenopsis* from the proliferating proembryoids of the explants which were selected in regeneration medium containing 100µM/ ml kanamycin after 30 day culture period. Belarmino and Mii (2000) transferred the explants in to selection media, hygromycin at 50 mg l^{-1} concentration.

Knapp *et al.* (2000) selected for transformants using bialaphos. PLBs which proliferated on selection medium containing 3 mg l^{-1} bialaphos were selected as transformants. In *Phalaenopsis*, Chia *et al.* (2002) carried out selection on regeneration medium containing 3 mg l^{-1} hygromycin for two months. Liau *et al.* (2003) reported the transformation of *Oncidium* using the selection medium 5 mg l^{-1} hygromycin. Chan *et al.* (2003) optimized the hygromycin concentration for the selection of *Phalaenopsis* transformants with 50 mg l^{-1}. Men *et al.* (2004) reported the use of selection medium supplemented with 30 mg l^{-1} hygromycin. Mishiba *et al.* (2005) reported the use of 20 mg l^{-1} hygromycin as the selection agent.

2. 5. 1. 10. Polymerase Chain Reaction (PCR) of Transformants

Confirmation of the putative transformants is usually done by PCR using the primer designed for the gene.

Kuehnle and Sugii (1992) reported the PCR analysis of the transgenic plants of *Dendrobium* Jaquelyn Thomas showing the integration of both *neo* and *gus A* fragments. Similar observations were also reported in *Dendrobium* White Angel (Chia *et al.*, 1994). In *Phalaenopsis,* Belarmino and Mii (2000) and Chia *et al.* (2002) confirmed the presence of transgene by PCR analysis. In *Dendrobium* the presence of transgene was assessed by PCR analysis (Yu *et al.*, 2001). Successful transformation in Cymbidium was confirmed by PCR analysis of transformants (Chen *et al.*, 2002).

The transformants for gene stacking in *Phalaenopsis* orchid to enable dual tolerance to pathogen attack was confirmed by PCR (Chan *et al.*, 2005). The transformed Cymbidium orchid plant was confirmed by PCR analysis (Yang *et al.*, 1999). Yu *et al.* (2001) confirmed the presence of putative transformants for 1knox gene DOH1 by PCR analysis. Specific oligonucleotide primers that amplify the 700 bp fragments spanning the 3'end of the CaMV 35S promoter and 5'end of the inserted *DOH1* antisense gene PCR amplification was performed with 30 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min.

Genetic transformation of two species of orchid was confirmed by PCR analysis (Men *et al.,* 2004). Mishiba *et al.*, (2005) confirmed *Agrobacterium*-mediated transformation of *Phalaenopsis* was by PCR analysis.

2. 5. 1. 11. Southern hybridization

Kuehnle and Sugii (1992) confirmed the putative transformants of *Dendrobium* using Southern blotting. Yu *et al.* (1999) confirmed transformation

and integration of transgenes in the protocorms of orchid (*Dendrobium* hybrid) by GUS histochemical assay and Southern blot hybridization.

Belarmino and Mii (2000) reported confirmation of successful transformation by histochemical GUS assay, PCR analysis and Southern hybridization of transformants.

Liau *et al.* (2003) obtained 28 independent transgenic orchid plants from which six transgenic lines that were positive for beta-glucuronidase were randomly selected and confirmed by Southern, northern and western blot analyses.

Men *et al.*, 2004 reported the integration and expression of the transgenes of two species of orchids were by confirming with Southern hybridization. You *et al.* (2003) obtained a total of 32 independent transgenic orchid lines out of which nine transgenic lines (beta-glucuronidase positive) were randomly selected and confirmed by Southern and northern blot analyses.

Chan *et al.* (2005) reported transgene integration in *Phalaenopsis* lines by confirming with Southern blot analysis. Mishiba *et al.* (2005) reported confirmation of transgene integration of hygromycin resistant orchids by Southern blot analysis.

Materials and Methods

3. MATERIALS AND METHODS

The experiment on "*Agrobacterium tumefaciens* mediated genetic transformation in orchid *Dendrobium* Sonia 17 with 1- aminocyclopropane1-carboxylic acid (ACC) synthase antisense gene" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during January 2004 to October 2006. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3. 1 SOURCE OF EXPLANT

The *in vitro* raised protocorm like bodies (PLBs) were used as the source explants for the study. PLBs are somatic protocorms derived from the *in vitro* culture of apical or axillary bud meristems. Two year old shoot apices of *Dendrobium* Sonia 17 were used for the generation of PLBs. Primary PLBs are induced by culturing the apical meristems tips. Secondary PLBs are formed on the surface of the primary PLBs. Proliferating PLBs are observed 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), Mumbai. The antibiotics and plant growth substances were purchased from Hi-Media Laboratories, Mumbai.

3. 2. 2 Glassware, Plastic ware and Other Materials

Borosilicate glassware and disposable sterile Petri dishes were purchased from Tarsons, Kolkata. The membrane filters used were from Sartorius, Germany.

3. 2. 3. Composition of Media

Basal MS medium (Murashige and Skoog, 1962) supplemented with various plant growth substances, benzyl adenine (BA), α-naphthalene acetic acid (NAA) and organic supplements like coconut water were used for plant tissue culture experiments (Appendix I).

For maintenance of *Agrobacterium* strain GV3101, Yeast Extract Mannitol (YEM) medium and AB minimal medium were used (Appendix II).

3. 2. 4. Preparation of Medium

Standard procedures were followed for the preparation of the plant tissue culture media (Murashige and Skoog, 1962). The pH of the medium was adjusted to 5.7 using 0.1N NaOH / HCl. Agar 6.3 g l^{-1} was added and the medium was heated to dissolve it. The medium was then dispensed into culture vessels at the rate of 15 ml per culture tube and 50 ml per conical flask. The test tubes and conical flasks were plugged with non absorbent cotton and sterilized by autoclaving under steam at a pressure of 1.06 kg cm^{-2} and a temperature of 121^0 C for 20 min (Dodds and Roberts, 1982). The medium was cooled to room temperature and stored in culture room at 25 ± 2^0 C.

For bacterial culture, the YEM and AB minimal medium were used. The pH of medium was adjusted to 6.9 and agar 15 g l^{-1} was added. The medium was

sterilized by autoclaving for 20 min at a pressure of 1.06 kg cm^{-2} and at 121^0 C. The medium was prepared in conical flask and stored in culture rooms at 25 ± 2^0 C.

3. 2. 5. Preparation of Stock Solution of Antibiotics

3. 2. 5. 1. Kanamycin

A stock solution (10^4 mg l^{-1}) was prepared by dissolving kanamycin monosulphate in sterile distilled water. It was then filter sterilized and stored at - 80^0 C (ultra low temperature freezer, Sanyo, Japan).

3. 2. 5. 2. Cefotaxime

A stock solution (10^4 mg l^{-1}) was prepared by dissolving cefotaxime in sterile distilled water. It was then filter sterilized and stored at - 80^0 C (ultra low temperature freezer, Sanyo, Japan).

3. 2. 5. 3. Rifampicin

A stock solution (10^4 mg l^{-1}) was prepared by dissolving rifampicin first in ethanol and then in sterile distilled water. It was then filter sterilized and stored at - 80^0 C (ultra low temperature freezer, Sanyo, Japan).

For the antibiotic sensitivity studies, the required concentrations of kanamycin and cefotaxime were diluted from the stock and added to the sterilized molten medium at 40°C.

***3. 2. 6. Agrobacterium* Strain**

Agrobacterium tumefaciens, GV3101 harbouring the plasmid pA_2A_4AB was used for the study.

3. 2. 6. 1. Vector

The vector pA_4A_2AB contains the ACS_2 (ACC synthase antisense) and the *nptII* (kanamycin resistance) gene under the control of ACS_4 promoter (Appendix VI).

3. 2. 6. 2. Maintenance of Agrobacterium Strain

The *Agrobacterium tumefaciens* strain, GV3101 harbouring the vector, pA_4A_2AB was grown on YEM medium supplemented with kanamycin 50 mg l^{-1}, rifampicin 50 mg l^{-1} and gentamycin 50 mg l^{-1}.

3. 3. INOCULATION AND OTHER ASEPTIC MANIPULATIONS

All the aseptic procedures were carried out in a laminar air flow cabinet (Thermadyne).

3. 4. CULTURE CONDITIONS

The cultures were incubated at 25 ± 2^0 C in air-conditioned culture room with 16 hr photoperiod (1000 lux) supplied by cool white fluorescent tubes. Relative humidity in the culture room varied between 50 and 60 per cent.

3. 5. PRODUCTION OF PROTOCORM LIKE BODIES

Primary protocorm like bodies were induced by culturing shoot apices. Shoots were collected and the shoot apices were cut out and washed in running tap water, followed by washing in sterile distilled water containing a few drops of Laboline. Rinsing with four or five changes of sterile distilled water. Then the explants were trimmed using sterile blade and taken to the laminar airflow chamber and surface sterilized with different concentrations of mercuric chloride.

Mercuric chloride was washed off by rinsing with four changes of sterile distilled water. Then the explants were transferred to sterile filter paper for absorbing excess moisture. The meristems were inoculated in culture bottles containing half strength MS semi solid medium supplemented with sucrose 30 g $l^{-1}$and agar 6.3 g l^{-1}. Different concentrations of organic supplement like coconut water were tried. The effect of different concentrations of activated charcoal and growth regulators on *in vitro* proliferation was also studied. The cultures were incubated at 25 ± 2^0 C in culture room with 16 hr photoperiod (1000 lux). Observations were recorded on the number of days taken for initiating PLBs and number of cultures initiating PLBs.

3.5. 1. Proliferation of Protocorm Like Bodies

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 25 ± 2^0 C and sub cultured for effective growth.

3. 6. 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 process after transformation. The PLBs produced on MS medium were transferred to Petri plates containing medium of same composition with different concentrations of kanamycin (0, 5, 25, 50, 75, 100, 125, 150, 200, 300, 400 and 500 mg l^{-1}) and cefotaxime (0, 5, 25, 50, 75, 100, 125, 150, 200, 300, 400 and 500 mg l^{-1}). The Petri plates were sealed with parafilm and kept at 25 ± 2^0 C. The culture response of the explants to varying concentrations of kanamycin and cefotaxime was evaluated for eight weeks.

The following scoring method was used for evaluation.

Score	Culture response
++++	Fully green
+++	Partially discolored
++	Bleached
+	Turning brown and dead

3. 8. SCREENING OF AGROBACTERIUM STRAIN FOR SENSITIVITY TO ANTIBIOTICS

Agrobacterium strain GV3101 was transferred to YEM medium in Petri plates with different concentrations of kanamycin or cefotaxime (5, 25, 50, 75, 100, 125, 150, 200, 300, 400 and 500 mg l^{-1}). The plates were sealed with parafilm and incubated at 28° C. Bacterial growth was observed for two days.

3. 9 GENETIC TRANSFORMATION OF DENDROBIUM SONIA

3. 9. 1. Preparation of *Agrobacterium* Suspension for co-cultivation

The *Agrobacterium tumefaciens* strain GV3101 harboring the vector, pA_4A_2AB was grown in Petri plates containing AB minimal medium with kanamycin 50 mg l^{-1} at 28^0 C overnight. *Agrobacterium* suspension for co-cultivation was prepared by picking a single colony from the plate and inoculating into 20 ml AB broth supplemented with antibiotics like kanamycin 50 mg l^{-1} and rifampicin 50 mg l^{-1}. AB broth with the bacterial strain was kept in a shaker overnight at 28°C at 10 rpm. The next day the culture was spun in a centrifuge at 5000 rpm at 4°C for 5 min. The pellets obtained were resuspended in 1ml of half

strength MS broth (with 200 μM acetosyringone) for co-cultivation. The overnight grown culture was diluted to1:5 with the fresh medium and the optical density (OD) of the culture were measured at 600 nm in a UV-Visible spectrophotometer (Spectronic Genesys 5) and the bacterial concentration was adjusted to 0.1 by dilution.

3. 9. 2. Preparation of Plant Material

The PLBs of *Dendrobium* were used as explants for co-cultivation. The PLBs were pre-cultured on half MS medium containing 0.2 mg l^{-1} BA for 15 days before co-cultivation to maintain cells in active division stage.

3. 9. 3. Co-cultivation

The co-cultivation was done in a laminar airflow cabinet. The PLBs pre-cultured for 15 days were pierced with needle to facilitate wounding and infection process. The PLBs were placed in sterile Petri plates and wetted with liquid half MS medium to avoid drying of explants. The explants were then mixed thoroughly with the prepared *Agrobacterium* suspension containing 100 μM and 200 μM of acetosyringone by gentle swirling for 15 and 20 min to standardise the optimum time required for the infection. The explants were blot dried with sterile filter paper and transferred to a Petri plate containing solidified half strength MS medium. Petri plates were sealed with parafilm and kept for co-cultivation in dark for three to four days at 28° C. The effect of number of days of co-cultivation on maximum transformation efficiency was standardized.

3. 9. 4. Incubation on Bacteriostatic Medium

After co-cultivation, PLBs were washed in half strength liquid MS medium containing 50 mg l^{-1} cefotaxime to kill the bacteria. The tissues were blot dried with sterile filter paper and transferred to sterile Petri plates containing half MS

medium supplemented with 50 mg l^{-1} cefotaxime for eradication of the remaining *Agrobacterium.*

3. 9. 5. Selection of Transformed Tissues

The transformed tissues were selected on MS medium containing kanamycin (200 mg l^{-1}) and cefotaxime (50 mg l^{-1}). The bacteriostatic agent was added in the initial subcultures. ThePLBs were maintained by subculturing once in seven days in the same medium. The transformed PLBs were maintained for eight weeks till the transformed tissues developed green sprouts from the cut edges. Amplification reaction was done with PLBs using the primers specific for *nptII* and ACS_2 (ACC synthase antisense) gene.

3. 10. Confirmation of Transformation

3. 10. 1 Isolation of genomic DNA

Total genomic DNA was isolated using modified protocol of Murray and Thompson (1980). PLBs (0.2 g) were taken and washed in distilled water and blot dried. Then the PLBs were ground into fine powder using liquid nitrogen with an autoclaved mortar and pestle. The powder was then transferred quickly to a 2ml eppendorf tube. To the tube 1.5 ml of pre warmed Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer was added. The tube was incubated at 65^0 C for 30 min with occasional mixing. The mixture was cooled to room temperature and centrifuged at 5000rpm for five min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and mixed well to get an emulsion by inverting the tube several times for 15 min. It was centrifuged at 10,000 rpm for 10 min and the aqueous phase was taken. To this equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 min. To the aqueous phase 2.5 volume absolute alcohol was added and mixed carefully. It was kept at -20^0C for 30 min. The

precipitated DNA was pelleted at 10,000 rpm for 10 min in a refrigerated centrifuge (Appendix III). The pellet was washed twice using 70 per cent ethanol and air dried. The pellet was dissolved in 50µl Tris EDTA (Ethylene Diamene Tetra Acetic acid) buffer (pH 8.0).

3. 10. 2 Quantification of DNA

Quantification of DNA was carried out with UV- Visible spectrophotometer (Spectronic Genesys 5). The optical density of the DNA samples were recorded at both 260 and 280 nm wave lengths. The concentration of DNA was found using the following formula:

Amount of DNA (µg/ 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 and 280 nm. The A_{260} /A_{280} value between 1.6 and 1.8 indicate the best quality of DNA.

3. 10. 3 Polymerase Chain Reaction (PCR)

The PCR analysis was performed with DNA isolated from the regenerated PLBs. A standard PCR mix was prepared for 20 µl total volume containing 20-30 ng of template DNA, 1 mM of each deoxyribonucletide, 10 pM of each primer, I unit of *Taq* DNA polymerase and 10X PCR buffer.

The genomic DNA of the transformed and the non-transformed plants obtained were amplified with the gene specific primers for ACC synthase antisense (*ACS2*) gene and *nptII* gene.

The following conditions were provided for the amplification of the specific regions in the genomic DNA in a Thermal cycler (PTC-150 mini cycler).

		ACS_2		*nptII*	
Step	**Stage**	**Temperature (^{0}C)**	**Duration**	**Temperature (^{0}C)**	**Duration**
1.	Initial Denaturation	94	5 min	94	3 min
2.	Denaturation	94	20 sec	94	30 sec
3.	Annealing	53	20 sec	52	30 sec
4.	Extension	72	1 min	72	1 min
5.	Final Extension	72	7 min	72	7 min

The step 2-4 was allowed to repeat 29 times.

3. 10. 3. 1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit. Required amount of agarose (1.0 %) was weighed out 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 (10^{-3} mg ml^{-1}) was added. The mixture was then poured to a pre set template with appropriate comb. After the gel was set the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank and was flooded with 1 X TAE buffer, to about 1mm above the gel. Required volume of DNA sample and gel- loading buffer (6 x loading dye *i.e.,* 40 per cent sucrose and 0.25 per cent bromophenol blue) were mixed. One of the wells was loaded with 5µl of the 100 bp molecular

weight marker with required volume of gel loading buffer. Electrophoresis was carried out at 50 volts until the loading dye reached three fourth of the length of the gel. The gel was documented using the gel doc (BIO-RAD).

3. 10. 4 Southern Hybridisation

Southern blotting and hybridisation were performed according to the standard protocol by Sambrook *et al.* (1989).

3. 10. 4. 1 Restriction digestion of genomic DNA

Genomic DNA (20 µg), each from transformed and non- transformed plant was taken for digestion with two enzymes, *Sca*I and *Xba*I. The digestion mixture was of 30 µl with the following constituents:

Sl. No.	Constituents	Quantity (µl)
1.	DNA	16.26
2.	Buffer	3.0
3.	*Sca* I	1.0
4.	*Xba* I	1.0
5.	BSA	3.0
6.	Deionized sterile water	5.74
	Total	30.0

Equal concentration of DNA was taken in both the cases. The reaction mixture was incubated at 37° C over night.

3. 10. 4. 2 Preparation of positive control

3. 10. 4. 2. 1 Isolation of the Plasmid DNA

A single colony of bacteria was inoculated in a 5 ml of LB broth taken in a 50 ml test tube and incubated for 12-16 hours at 28°C. The broth was transferred into microfuge tubes and centrifuged for 5 min at 10, 000 rpm. The supernatant was discarded and the tube was inverted on a paper towel and blotted. 100 µl of ice cold solution I (Appendix IV) was added and the cells were resuspended in it by vigorous vortexing. This was followed by the addition of 200 µl of freshly prepared solution II (Appendix IV) and the contents were mixed by inverting it 4 times. The tube was then incubated for five minutes in ice. 150 µl of ice-cold solution III (Appendix IV) was added. The tube was incubated at room temperature for five minutes. The bacterial lysate was centrifuged at 12, 000 rpm for five minutes. The cleared lysate was transferred to an eppendorf tube. Equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added and the content was mixed well. The contents were centrifuged at 12, 000 rpm for five min to separate the aqueous layer. To the aqueous layer equal volume of chloroform: isoamyl alcohol (24:1) was added and the contents were centrifuged at 12, 000 rpm for five min. The acqueous layer was separated and double volume of 100 per cent ethanol and $1/10^{th}$ volume of 3M sodium acetate was added. The contents were stored at –20°C for one hour and were spun at 5, 000 rpm for five minutes to collect the pellet. The pellet was washed with 70 per cent ethanol air dried and dissolved in 1X TE and stored at 4°C.

3. 10. 4. 2. 2 PCR of the Plasmid DNA

The plasmid DNA was subjected to polymerase chain reaction, to get the gene specific amplification. A standard PCR mix was prepared for 20 µl final volume. The reactions were set up with 20 ng of template DNA, 1 mM of each dNTPs, 10 pM of each primer, I unit of *Taq* DNA polymerase and 1X PCR buffer.

The plasmid DNA obtained was amplified with the gene specific primers for ACC synthase antisense (*ACS_2*) gene. The PCR mix was for a total volume of 20 µl and consisted of template DNA based on the concentration of each sample, 2 µl of 10X buffer and dNTPs, 0.5 µl each of forward and reverse primer and 0.25 µl of *Taq* DNA polymerase were added. The final volume was made up with sterile water.

The following conditions were provided for the amplification of the specific regions in the plasmid DNA in a Thermal Cycler(PTC-150 mini cycler).

Step	Stage	Temperature (°C)	Duration
1.	Initial Denaturation	94	5 min
2.	Denaturation	94	30 sec
3.	Annealing	53	20 sec
4.	Extension	72	1 min
5.	Final Extension	72	7 min
6.	Storage	4	24 hours

3. 10. 4. 2. 3 Elution of the PCR product and Gel Band Purification

GFX™ PCR DNA and Gel Band Purification kit of Amersham Pharmacia Biotech. Inc. USA was used for the elution of the bands from the gel.

The PCR product was cut from the gel and weighed to the nearest 10 mg in a 1.5 ml microfuge tube of known weight. 10 µl of the capture buffer was added for each 10 mg of the gel slice. The tube was mixed vigorously by vortexing and was placed in a water bath at 60°C until the agarose got dissolved completely. A brief centrifugation was given to collect the sample at the bottom of

the tube. The sample was then transferred to GFX^{TM} column placed in a collection tube and incubated at room temperature for one minute. Then it was centrifuged at 10,000 rpm for 30 sec. The flow through was discarded by emptying the collection tube and the GFX^{TM} column was again placed in the collection tube. Wash buffer (500 µl) was added to the column and centrifuged at 12,000 rpm for 30 sec. The collection tube was then discarded and the column was placed in a new sterile microfuge tube. Nuclease free water (50 µl) was added directly on the top of the glass filter and incubated at room temperature for one minute. The tube was then centrifuged at full speed for one minute to recover the purified DNA.

The amplified DNA was quantified in spectrophotometer and diluted to 50 ng/µl and was used as the positive control. The restricted reaction mixture was loaded in 1 per cent agarose gel along with 1 µl of plasmid DNA (positive control). The 100 bp ladder and 1kb ladder were also loaded in the agarose gel. The electrophoresis was done at 30 V over night. The resultant smear of digested fragments was transferred to nylon membrane.

3. 10. 4. 3 Transfer to nylon membrane

The agarose gel was placed in a tray containing depurination solution with the entire gel being submerged in the solution. The tray was incubated for five min with gentle rocking. The solution was discarded and replaced with denaturation solution in similar way for 10 min. This was followed by the neutralization solution for another 10 min of incubation.

The gel was blotted to nylon membrane by vacuum suction method. A wet Whatman No 2 filter paper was placed on the top of the tray over which the nylon membrane and gel were placed. A polythene sheet with a window was placed over the membrane. This covered the entire tray from outside contact. Then a perfect vacuum was created to suck out air by pump. In this process the DNA from the gel was brought down to the membrane. The tray was filled with 1 X SSC and a

suction pressure of five psi was produced for one hour to complete the entire transfer of DNA from the gel to the membrane.

The denatured DNA that has been transferred to the membrane was cross-linked with the help of UV cross linker by a two minute exposure to UV. The DNA was bound covalently to the membrane and this was used for hybridisation with the labeled probe.

3. 10. 4. 4 Preparation of probe

The probe was labeled used NE blot kit (Amersham). The DNA (25-100 ng) was taken and made up to 33µl volume using nuclease free water. The DNA was denatured in boiling water bath for five min and snap cooled in ice for another five min. The DNA was centrifuged briefly in cold and 5µl of 10X labeling buffer was added. 2µl each of dNTPs (dATP, dTTP, dGTP) was added to the mixture. 1µl of the DNA polymerase Klenow fragment was added for the extension of the probe to which the radio labeled dCTP is added. The mixture was incubated at 37°C for one hour. The reaction was terminated by adding 5µl of 0.2M EDTA (pH 8.0).

3. 10. 4. 5 Purification and use of labeled probes

Probes synthesized had to be separated from unincorporated nucleotides. The purification was done by denaturing by heating in boiling water bath at 95-100°C for five min and placing in ice bath for five min. The labeled DNA was used directly in hybridisation.

3. 10. 4. 6 Hybridisation

The blot membrane was made wet with 2X SSC and inserted into the hybridisation tube without any bubbles. Pre hybridisation buffer (40 ml) and 2µl

BSA was added to the blot. Then the blot was run at a temperature of 65°C for two hour as a prehybridisation treatment in the hybridisation solution. The BSA was bound to the membrane in the free space left and thus effectively blocked the space from binding the probe in the subsequent hybridisation treatment.

The probe DNA was then added to the glass tube containing the membrane and left for hybridisation at a temperature of 65°C for 14 hours.

3. 10. 4. 7 Washing and exposure to photosensitive film

The membrane was taken out of the hybridisation solution and the solution was discarded. The membrane was then washed using the washing solution 1 (Appendix V) at room temperature for 10-15 min followed by two washes in washing solution 2 (Appendix V) at 45°C for 15 min each. As the radioactive emission count was very high final wash was given with washing solution 3 (Appendix V) at 65°C.

The excess solution adhered was dripped off and the slightly wet membrane was wrapped with saran wrap and exposed to an imaging screen (BIO-RAD), placed in a cassette overnight and the image was resolved by scanning (Molecular Imager Fx) the screen.

Results

4. RESULTS

The results of the experiments on *Agrobacterium tumefaciens* mediated genetic transformation in *Dendrobium* Sonia 17 carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during January 2004 to October 2006 are presented below.

4. 1 ESTABLISHMENT OF *IN VITRO* CULTURE

4.1.1 *In vitro* Establishment of PLBs

4.1.1.1 Surface Sterilization

Surface sterilization of the shoot tips with 0.1 per cent mercuric chloride for ten minutes was the most effective treatment with a culture establishment of 96.50 per cent (Table 1). The treatment with 0.1 per cent mercuric chloride for five minutes was 93.36 per cent. The sterilization with 0.08 percent of mercuric chloride for ten minutes was 78.69 percent and that for five minutes was 72.66 per cent.

4. 1. 1. 2 Effect of Coconut Water on in vitro Proliferation of PLBs

The addition of coconut water showed gradation in the establishment of PLBs. The highest establishment (88.80 %) was shown when 150 ml l^{-1} of coconut water was added (Table 2). The control recorded the least proliferation rate of 72.50 percentage (Fig. 1).

Plate 1. Protocorm like bodies of *Dendrobium* Sonia 17

Table 1. Effect of surface sterilants on establishment of shoot tip cultures

Sterilant	Concentration (%)	Duration of treatment (min)	Establishment (%)
Mercuric Chloride	0.08	5	72.66
		10	78.69
	0.10	5	93.36
		10	96.50

Table 2. Effect of Coconut Water on *in vitro* proliferation of Protocorm Like Bodies

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

Sl. No	Coconut water ml/l	Establishment (%)
1.	Nil	72.50
2.	100	82.90
3.	150	88.80
4.	200	81.20
5.	300	72.70

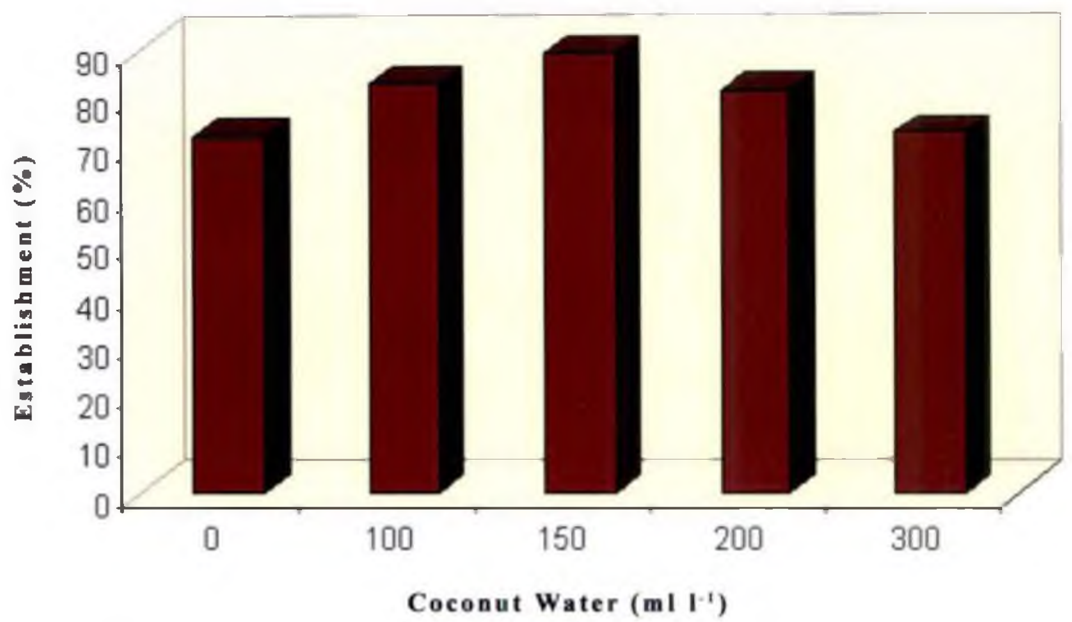


Fig. 1. Effect of coconut water on *in vitro* proliferation of protocorm like bodies

4. 1. 1. 3 Effect of Activated charcoal on in vitro Proliferation of PLBs

The highest establishment (87.60 %) was recorded with a concentration of 0.5 g l^{-1} of charcoal was added in the medium (Table 3). Lower proliferation rate was observed when charcoal was absent in the medium (Fig. 2).

4. 1. 1. 4 Effect of Growth Regulators on in vitro Proliferation of PLBs

The highest culture establishment (92.50 %) was recorded with BA 0.2 mg l^{-1} (Table 4). When BA 1.0 mg l^{-1} was used 90.20 per cent proliferation was obtained. When BA 1.0 mg l^{-1} and NAA 1.5 mg l^{-1} were used together the proliferation was 87.02 per cent. Without growth regulators the establishment was 85.55 per cent (Fig. 3).

4.2 SENSITIVITY OF PROTOCORM LIKE BODIES (PLBS) TO ANTIBIOTICS

The sensitivity of PLBs to different doses of antibiotics in half MS medium was tested.

4.2.1 Kanamycin

The sensitivity of PLBs to different doses of kanamycin (5-500 mg l^{-1}) was recorded. In the medium containing kanamycin 5 mg l^{-1}, the PLBs remained green up to six weeks, later they turned discoloured. The percentage survival of PLBs in kanamycin 5 mg l^{-1} after eight weeks was 62.6 per cent (Table 5).

In the media containing kanamycin 25 and 50 mg l^{-1}, the PLBs remained green up to five weeks. The percentage survival of the PLBs in kanamycin 25 and 50 mg l^{-1} after eight weeks were 52.7 and 49.2, respectively (Fig. 4).

Table 3. Effect of Activated charcoal on *in vitro* proliferation of Protocorm Like Bodies

Sl. No	Activated charcoal (g/l)	Establishment (%)
1.	Nil	66.80
2.	0.5	87.60
3.	1.0	75.00
4.	1.5	72.30
5.	2.0	70.90

Table 4. Effect of Growth Regulators on *in vitro* Proliferation of Protocorm Like Bodies

Sl. No	Growth Regulators (mg/l)	Establishment (%)
1.	NAA (1.5)+ BA (1.0)	87.02
2.	BA (1.0)	90.20
3.	BA (0.2)	92.50
4.	Nil	85.55

Table 5. Sensitivity of Protocorm Like Bodies to different doses of kanamycin

Sl No.	Kanamycin mg l^{-1}	Sensitivity (weeks)								Survival (%) (after 8 weeks)
		I	II	III	IV	V	VI	VII	VIII	
1	Nil	++++	++++	++++	++++	++++	++++	++++	++++	100.0
2	5	++++	++++	++++	++++	++++	++++	++++	++	62.6
3	25	++++	++++	++++	++++	++++	+++	+++	++	52.7
4	50	++++	++++	++++	++++	++++	++	++	++	49.2
5	75	++++	++++	++++	++++	++++	++	++	++	47.9
6	100	++++	++++	++++	++++	+++	++	++	++	44.2
7	125	++++	++++	++++	++++	+++	++	++	++	32.8
8	150	++++	+++	+++	+++	+++	++	++	++	25.6
9	200	++++	+++	+++	+++	++	++	++	+	20.0
10	300	+++	+++	+++	+++	++	++	+	+	5.9
11	400	+++	+++	+++	++	+	+	+	+	0
12	500	+++	+++	++	++	+	+	+	+	0

++++ - Fully green
+++ - Partially discoloured
++ - Bleached tissues
+ - Tissues turning brown and dead

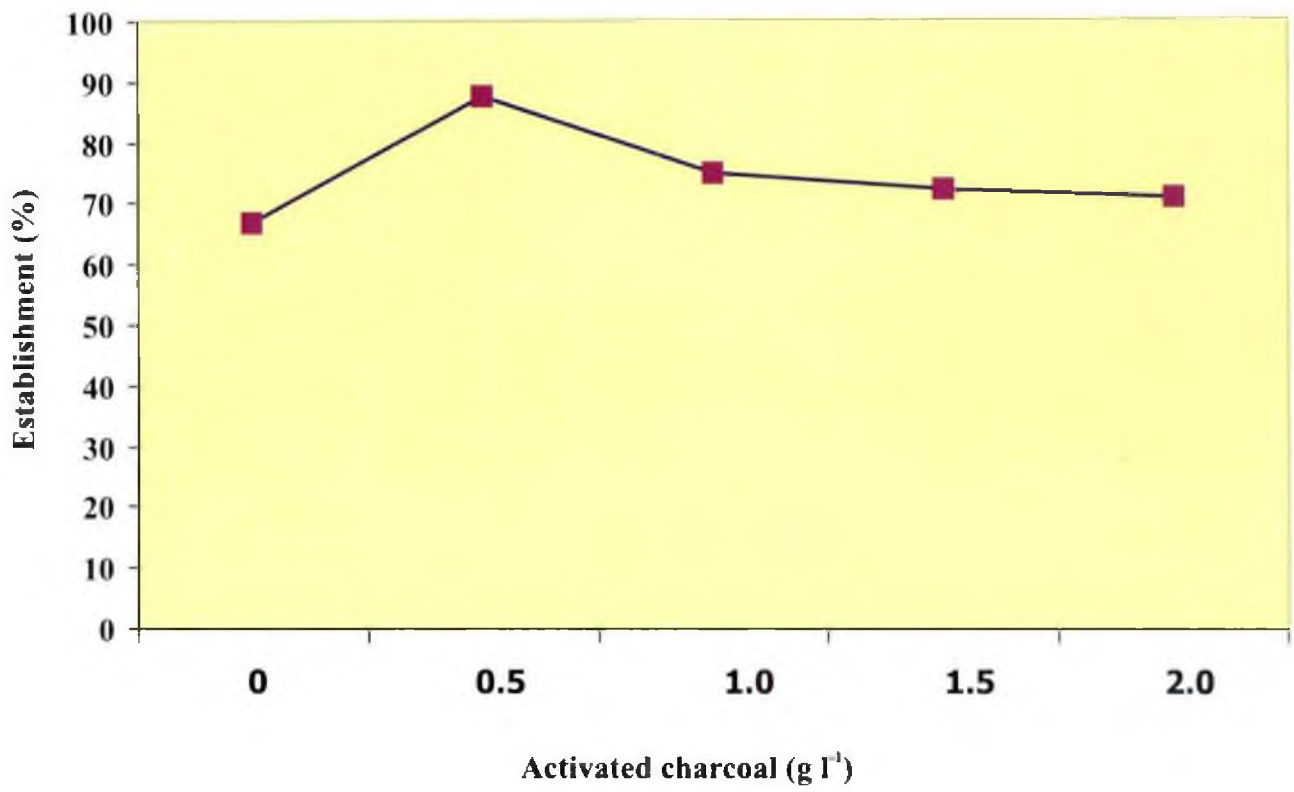


Fig. 2. Effect of activated charcoal on in vitro proliferation of protocorm like bodies

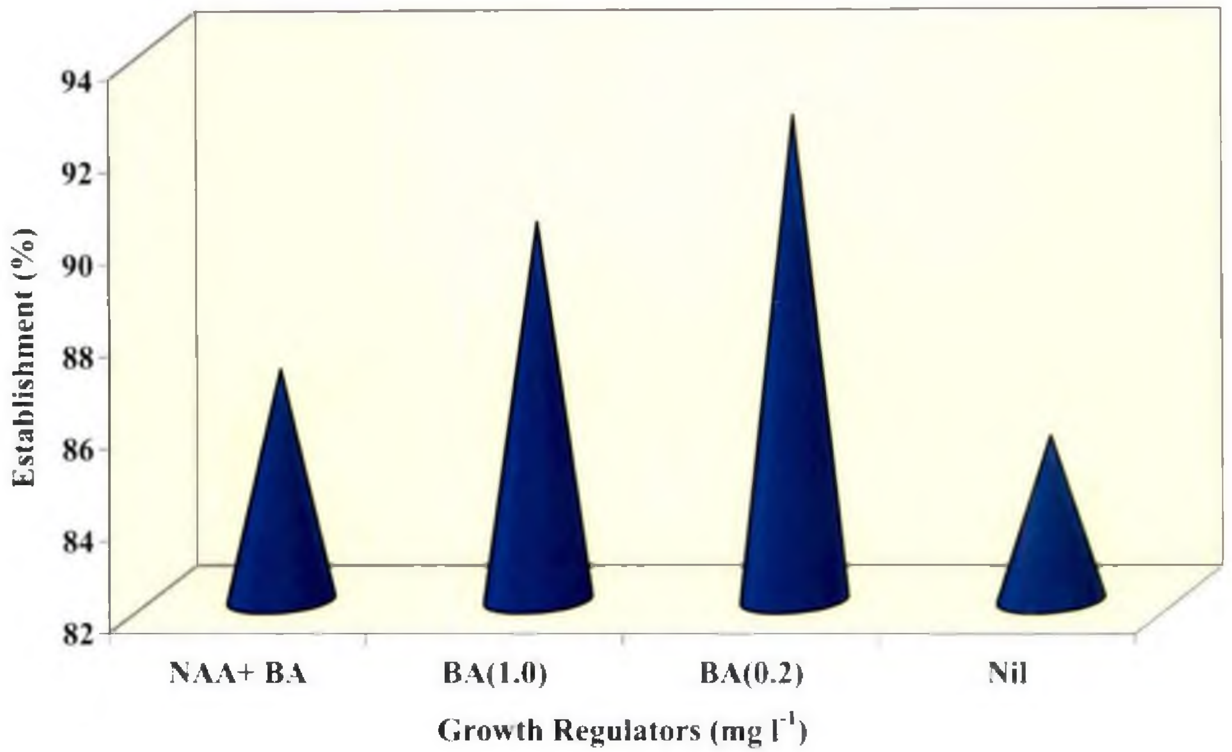


Fig. 3. Effect of growth regulators on *in vitro* proliferation of protocorm like bodies

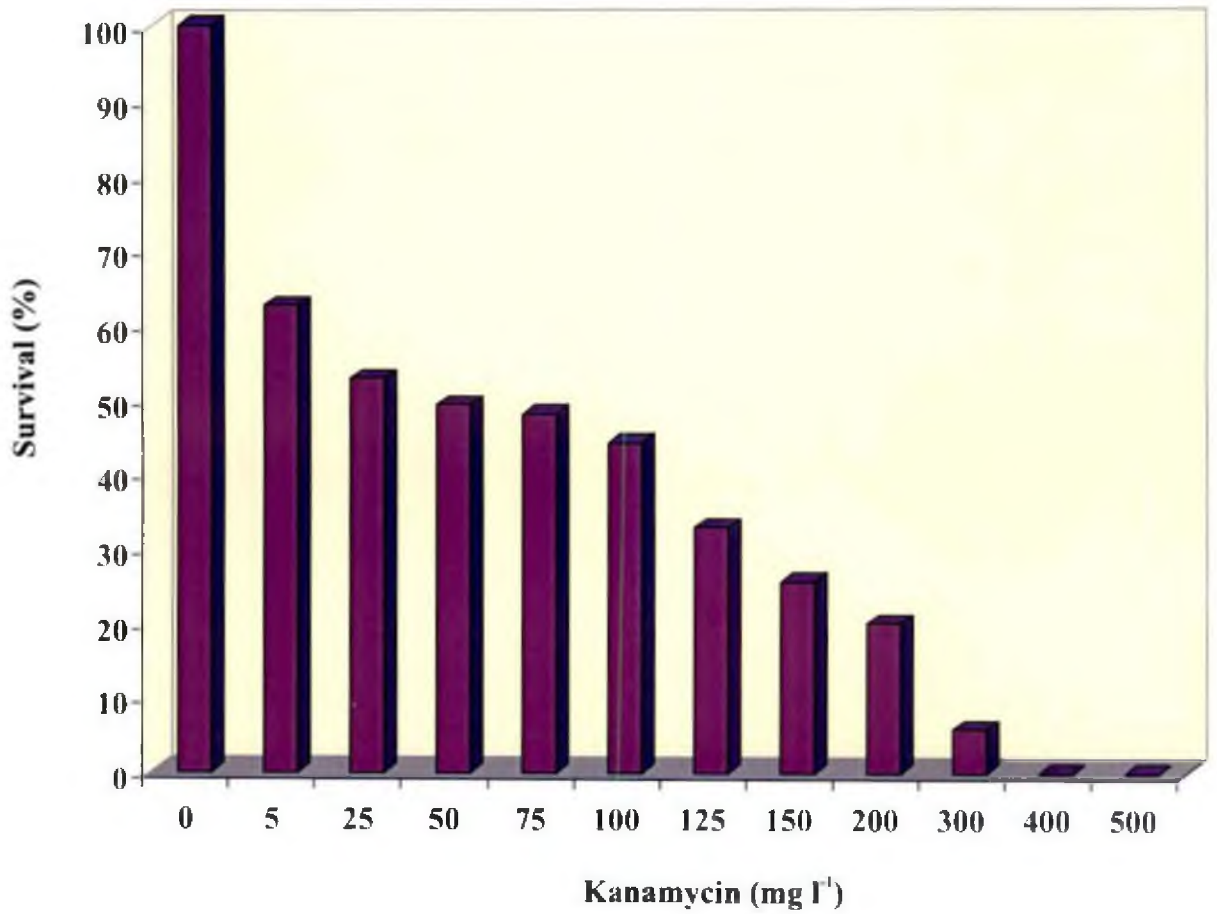


Fig. 4. Sensitivity of protocorm like bodies to different doses of kanamycin

The PLBs remained green up to five weeks in kanamycin 75 mg l^{-1} after inoculation. The PLBs were partially discoloured and bleached after six weeks of inoculation and remained fully bleached after seven weeks of inoculation. The percentage survival of callus after eight weeks was 47.9.

The PLBs remained green up to four weeks in medium containing 100 mg l^{-1} of kanamycin. They were partially discoloured after five weeks and bleached after six weeks of inoculation. The PLBs were completely bleached and turned whitish after six weeks of inoculation and the percentage survival of PLBs was found to be 44.2 after eight weeks.

In kanamycin, 125-150 mg l^{-1} the PLBs remained green only up to three weeks after inoculation. The PLBs became partially discoloured after two weeks and turned brown and dead after five weeks. After eight weeks the PLBs turned fully bleached and whitish and the per cent survival was 32.8 and 25.6 per cent, respectively.

The PLBs remained partially discoloured up to three weeks in medium containing 200 mg l^{-1} of kanamycin. The PLBs were completely bleached and turned whitish after four weeks of inoculation and the percentage survival of PLBs was found to be zero after eight weeks.

The tissues were green only up to one week after inoculation in kanamycin 300-500 mg l^{-1}. Later they became discoloured after two weeks and bleached after three weeks. After four weeks the tissues turned brown and dead. The percentage survival after eight weeks was zero.

The highest survival (62.6 %) was observed in kanamycin 5 mg l^{-1} and minimum survival percentage was observed in kanamycin 200 mg l^{-1}. For the initial screening of explants for the selection of putative transformants, kanamycin was used at a concentration of 250 mg l^{-1} (Plate 2).

(A) Kanamycin 5 mg l^{-1}

(B) Kanamycin 75 mg l^{-1}

(C) Kanamycin 100 mg l^{-1}

(D) Kanamycin 150 mg l^{-1}

(E) Kanamycin 300 mg l^{-1}

(F) Kanamycin 500 mg l^{-1}

Plate 2. Sensitivity of protocorm like bodies to different concentrations of kanamycin

4.2.2 Cefotaxime

In the medium containing cefotaxime 5 mg l^{-1}, the PLBs remained green up to seven weeks (Table 6). The survival of the callus after eight weeks was observed to be 70.0 per cent.

In cefotaxime 50 mg l^{-1}, the tissues remained green up to seven weeks after inoculation. They were partially discoloured after eight weeks. Only 68.9 per cent of the callus survived after eight weeks in 50 mg l^{-1} cefotaxime (Fig. 5).

The PLBs were green up to seven weeks, became partially discoloured after eight weeks, in the media containing cefotaxime 100 and 150 mg l^{-1}. The tissues remained bleached and whitish after eight weeks and the percentage survival of PLBs after eight weeks was 66.5 and 65.4, respectively.

The PLBs were green up to seven weeks, became partially discoloured and bleached after eight weeks in medium supplemented with cefotaxime 200-300 mg l^{-1}. The survival of PLBs after eight weeks was 60.7 and 50.8 per cent, respectively.

In cefotaxime 350 mg l^{-1} and 400 mg l^{-1}, the PLBs were green up to five weeks after inoculation, they turned partially discoloured after six weeks and bleached after seven weeks. The PLBs turned whitish and bleached after six weeks of culture and the survival of PLBs after eight weeks was 44.5 and 40.9 per cent, respectively.

The PLBs remained green up to four weeks in cefotaxime 450 mg l^{-1}. They were partially discoloured after six weeks and bleached after four weeks. The PLBs turned bleached after seven weeks and turned brown and dead after eight weeks of inoculation. In the media containing cefotaxime 500 mg l^{-1}, the PLBs

Table 6. Sensitivity of Protocorm Like Bodies to different doses of cefotaxime

Sl No.	Cefotaxime (mg l^{-1})	Sensitivity (weeks)								Survival (%) (after 8 weeks)
		I	II	III	IV	V	VI	VII	VIII	
1	Nil	++++	++++	++++	++++	++++	++++	++++	+++	100.0
2	5	++++	++++	++++	++++	++++	++++	+++	++	70.0
3	50	++++	++++	++++	++++	++++	+++	++	++	68.9
4	100	++++	++++	++++	++++	+++	++	++	+	66.5
5	150	++++	++++	++++	++++	+++	++	++	+	65.4
6	200	++++	++++	++++	+++	+++	++	++	+	60.7
7	250	++++	++++	++++	+++	+++	++	+	+	54.3
8	300	++++	++++	++++	+++	++	++	+	+	50.6
9	350	++++	++++	++++	+++	++	+	+	+	45.8
10	400	++++	++++	++++	+++	++	+	+	+	40.2
11	450	++++	++++	+++	++	+	+	+	+	0
12	500	++++	+++	++	++	+	+	+	+	0

++++ - Fully green
+++ - Partially discoloured
++ - Bleached tissues
\+ - Tissues turning brown and dead

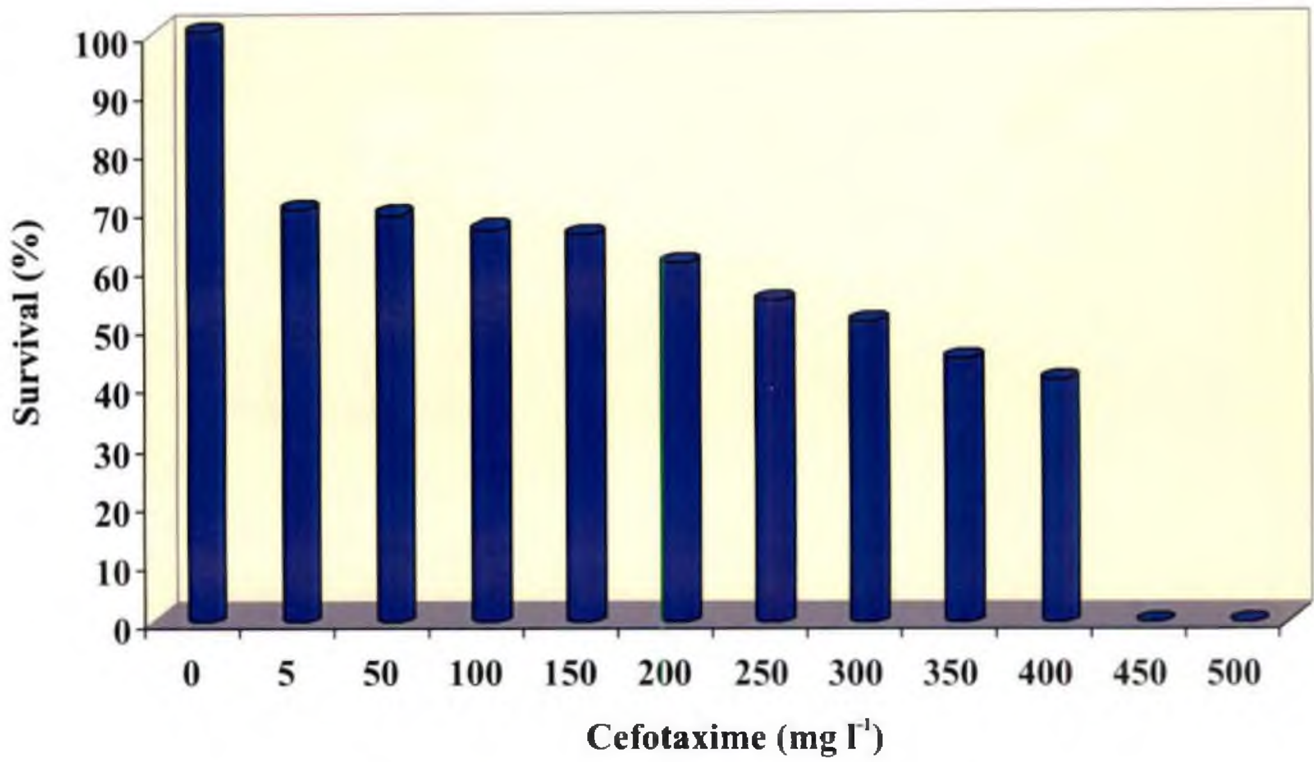


Fig. 5. Sensitivity of protocorm like bodies to different doses of Cefotaxime

were partially discolored after one week and they turned brown and dead after eight weeks (Plate 3).

The highest survival of 70 per cent was observed in cefotaxime 5 mg l^{-1} and minimum survival percentage was observed in cefotaxime 400 mg l^{-1}.

4.3 SENSITIVITY OF *AGROBACTERIUM* STRAIN TO ANTIBIOTICS

The sensitivity of the bacterial strain GV3101 harbouring pA_4A_2AB to different doses of antibiotics is presented below.

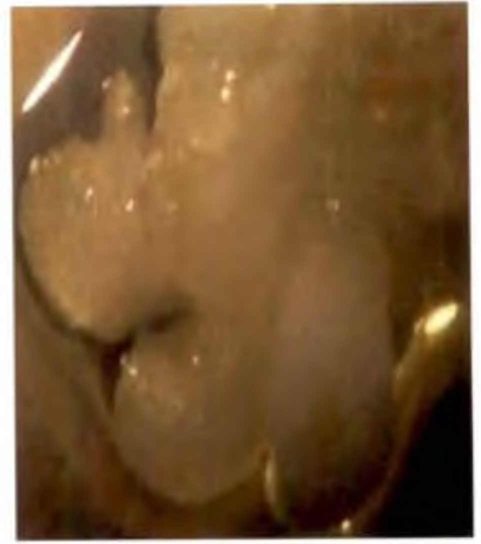
4.3.1 Kanamycin

The growth of *Agrobacterium* strains GV3101 harbouring the plasmid vector pA_4A_2AB in YEM medium containing different concentrations of kanamycin (5-500 mg l^{-1}) two days after culture inoculation was observed (Table 7). The bacterial cultures were resistant to kanamycin up to a concentration of 300 mg l^{-1}. No bacterial growth was observed in YEM medium containing kanamycin 350, 400 and 500 mg l^{-1}.

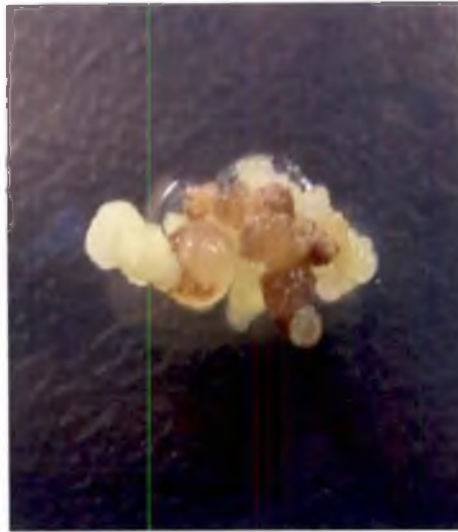
4.3.2 Cefotaxime

The growth of *Agrobacterium* strains GV3101 containing the plasmid vector pA_4A_2AB in YEM medium with different concentrations of cefotaxime (5-300 mg l^{-1}) after two days of culture was observed (Table 8). Bacterial growth was observed in YEM medium containing cefotaxime 5 to 25 mg l^{-1}. No bacterial growth was observed in YEM medium containing cefotaxime above 25 mg l^{-1} concentrations.

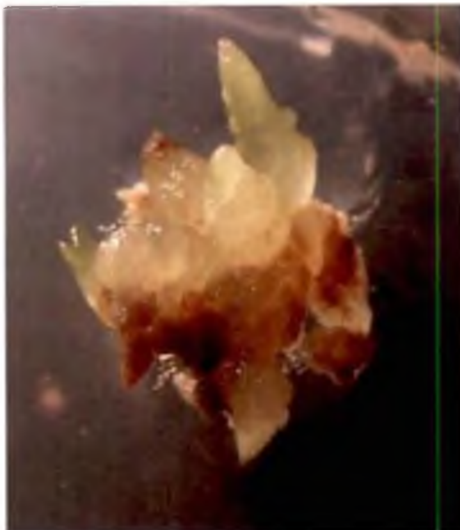
(A) Cefotaxime 5 mg l^{-1}



(B) Cefotaxime 75 mg l^{-1}



(C) Cefotaxime 100 mg l^{-1}



(D) Cefotaxime 300 mg l^{-1}

(E) Cefotaxime 500 mg l^{-1}

Plate 3. Sensitivity of protocorm like bodies to different concentrations of cefotaxime

Table 7. Sensitivity of *Agrobacterium tumefaciens* to kanamycin

Sl No.	Kanamycin (mg l^{-1})	Bacterial growth *
		GV3101
1	0	+
2	5	+
3	25	+
4	50	+
5	75	+
6	100	+
7	150	+
8	200	+
9	300	+
10	350	-
11	400	-
12	500	-

* Mean of three replications
\+ Bacterial growth
\- No bacterial growth

Table 8. Bacteriocidal activity of cefotaxime

Sl No.	Cefotaxime (mg l^{-1})	Bacterial growth*
		GV3101
1	0	+
2	5	+
3	25	+
4	50	-
5	75	-
6	100	-
7	125	-
8	150	-
9	200	-
10	250	-
11	300	-

* Mean of three replications
\+ Bacterial growth
\- No bacterial growth

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4.4 GENETIC TRANSFORMATION IN *DENDROBIUM* SONIA 17

4. 5. 1 Verification of the presence of ACS_2 and *nptII* gene in the plasmid

The presence of the ACS_2 and *nptII* gene in the vector sequence within transformed *Agrobacterium* colonies was confirmed by PCR of the plasmid DNA with ACS_2 and *nptII* gene specific primers. The amplified product had an expected size of 614 bp and 700 bp, respectively. Visual verification was done by comparing the PCR bands with 100 bp DNA marker (Plate 4, 5).

4. 5. 2 Effect of Infection Time

The effect of infection time on transformation efficiency was recorded. Infection was carried out for 15 and 20 min to standardize the optimum time required for transformation. Transformation was effective both in 15 and 20 min infection period. The transformation efficiency was 22.50 per cent in 15 min of infection period. As the infection time was extended to 20 min an increased transformation efficiency of 27.50 per cent was observed (Table 9).

4. 5. 3 Effect of Number of Days of Co-cultivation

Agrobacterium infected PLBs were co-cultivated at 28°C in a culture room in dark for three and four days. The effect of number of days of co-cultivation on the transformation efficiency was studied. The efficiency of transformation was found to be increased from 23.5 per cent to 28.5 per cent when the co-cultivation period was increased from three to four days. (Table 10).

4.5.4 Effect of Acetosyringone

Transformation efficiency could be increased by addition of acetosyringone (200 μM) to infection and co-cultivation medium. Transformation

Plate 4.

Lane 1- T1- *ACS_2* gene amplified from plasmid DNA of GV 3101

Lane 2- T2- *ACS_2* gene amplified from plasmid DNA of GV 3101

Lane 3- T3- *ACS_2* gene amplified from plasmid DNA of GV 3101

Lane 4- M- 100 bp DNA marker

Plate 5.

Lane 1- M- 100 bp DNA marker

Lane 2- T1- *nptII* gene amplified from plasmid DNA of GV 3101

Lane 3- T2- *nptII* gene amplified from plasmid DNA of GV 3101

Lane 4- T3- *nptII* gene amplified from plasmid DNA of GV 3101

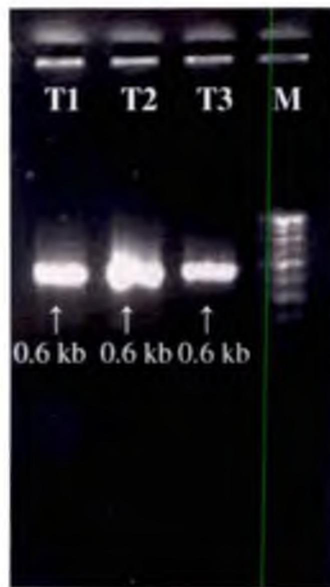

Plate 4. Verification for the presence of *ACS_2* gene in the plasmid DNA of GV3101 by PCR

Plate 5. Verification for the presence of *npt II* gene in the plasmid DNA of GV3101 by PCR

Table 9. Effect of infection time on transformation efficiency

Infection time (min)	Percent explants retained		Transformation efficiency (%)
	After co-cultivation	After incubation	
	GV3101	GV3101	GV3101
15	94.33	84.33	22.50
20	91.85	79.57	27.50

Table 10. Effect of co-cultivation time on transformation efficiency

No. of days of co- cultivation	Percent explants retained		Transformation efficiency (%)
	After co-cultivation	After incubation	
	GV3101	GV3101	GV3101
3	94.33	84.33	23.50
4	91.85	79.57	28.50

efficiency of 27.50 per cent was obtained when acetosyringone 200 µM was used in the infection and co-cultivation medium. In the absence of acetosyringone the transformation efficiency was only 17.30 per cent.

4.5.5 Survival of PLBs in Selection Medium

The survival of the PLBs in selection medium was observed after two weeks. The survival percentage was increased from 76.47 to 88.23 per cent when the kanamycin concentration in the selection medium was increased from 150 mg l^{-1} - 200 mg l^{-1} (Table 11; Plate 6, 7).

Transformation of the explants with *Agrobacterium tumefaciens*

Transformation was performed for the transfer of ACS_2 and *nptII* gene with the strain GV3101.

The success of the transformation of orchid with ACS_2 and *nptII* gene was confirmed with the following tests.

Identification of the transgenic plants

The PLBs were raised in antibiotic containing selection media for the confirmation of transgene.

Polymerase Chain Reaction (PCR)

The transgenes were confirmed by PCR using ACS_2 and *nptII* gene specific primers. Out of the three plant samples used for amplification two gave an appreciable quantity of the product at 614 bp and 700 bp, respectively. The PCR products and the positive control were of the same size (Plate 8, 9).

Table 11. Survival of tissues in selection medium

Kanamycin concentration (mg l^{-1})	Bacterial strain	No. of tissues remained green in selection medium	No. of tissues turned brown in selection medium	Survival of tissues in selection medium (%)
150	GV3101	15	2	88.23
200	GV3101	13	4	76.47

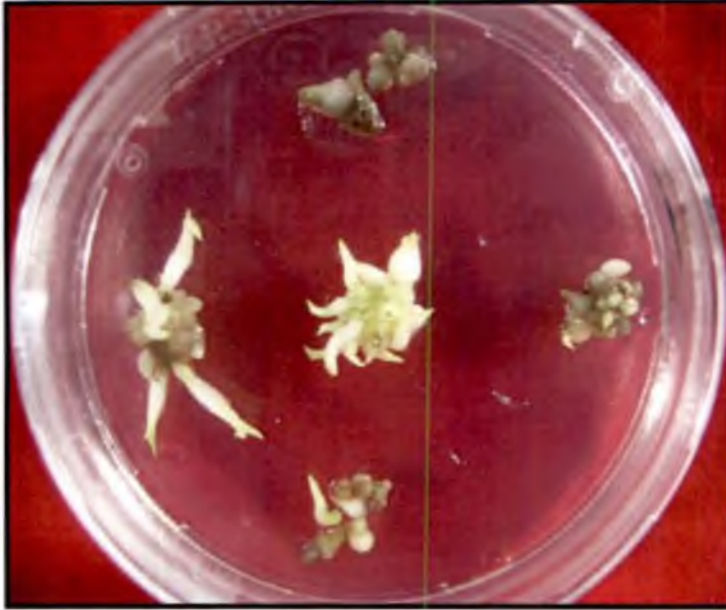

Plate 6. Protocorm like bodies in selection medium

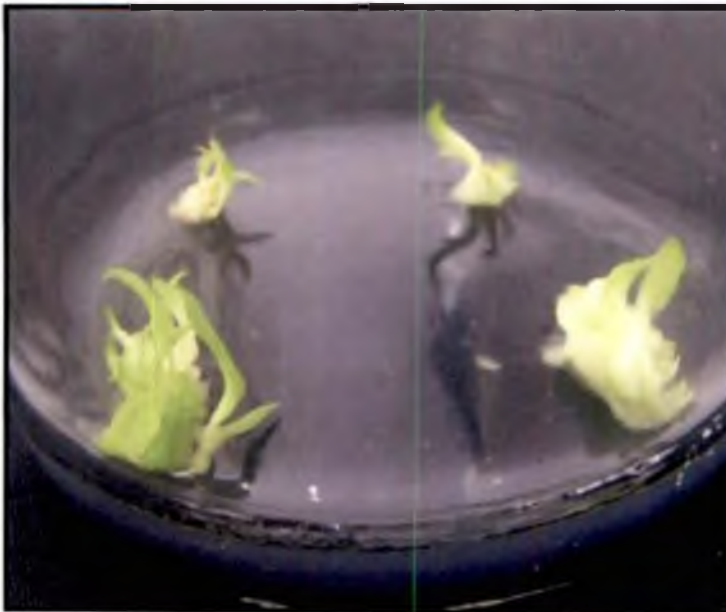

Plate 7. Transgenic tissues in selection medium

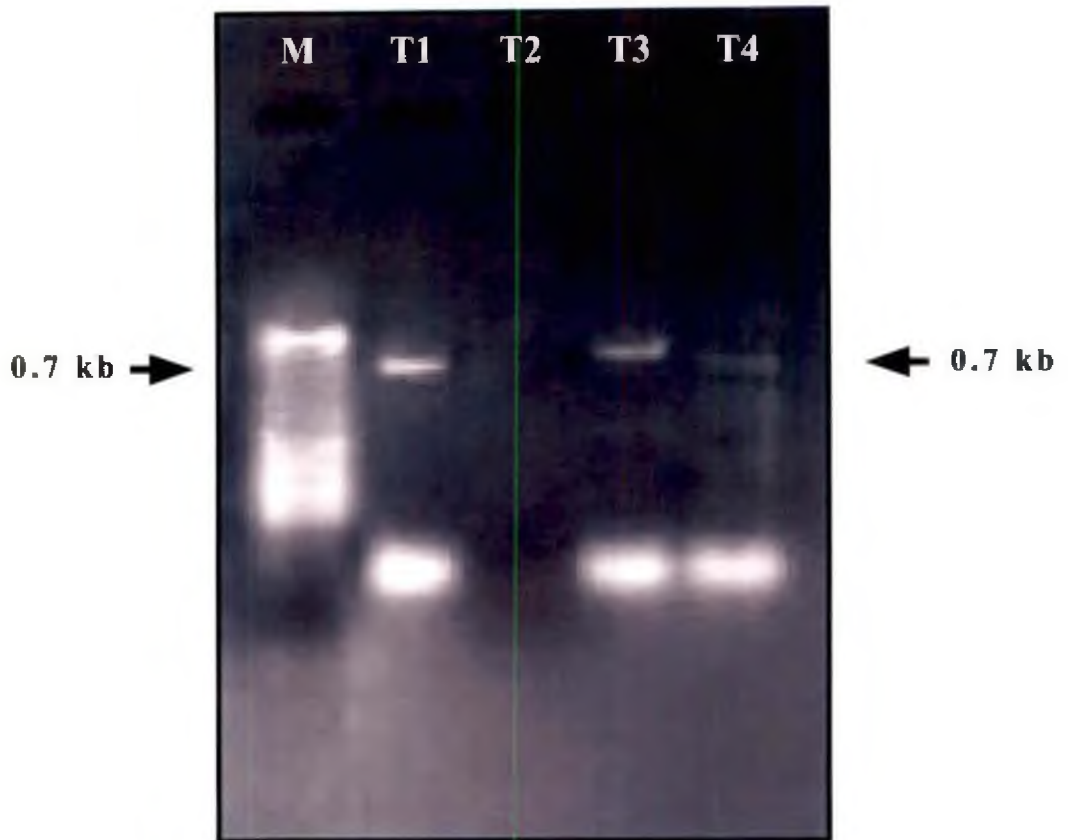

Plate 8. Confirmation of *npt II* gene in transformants by PCR

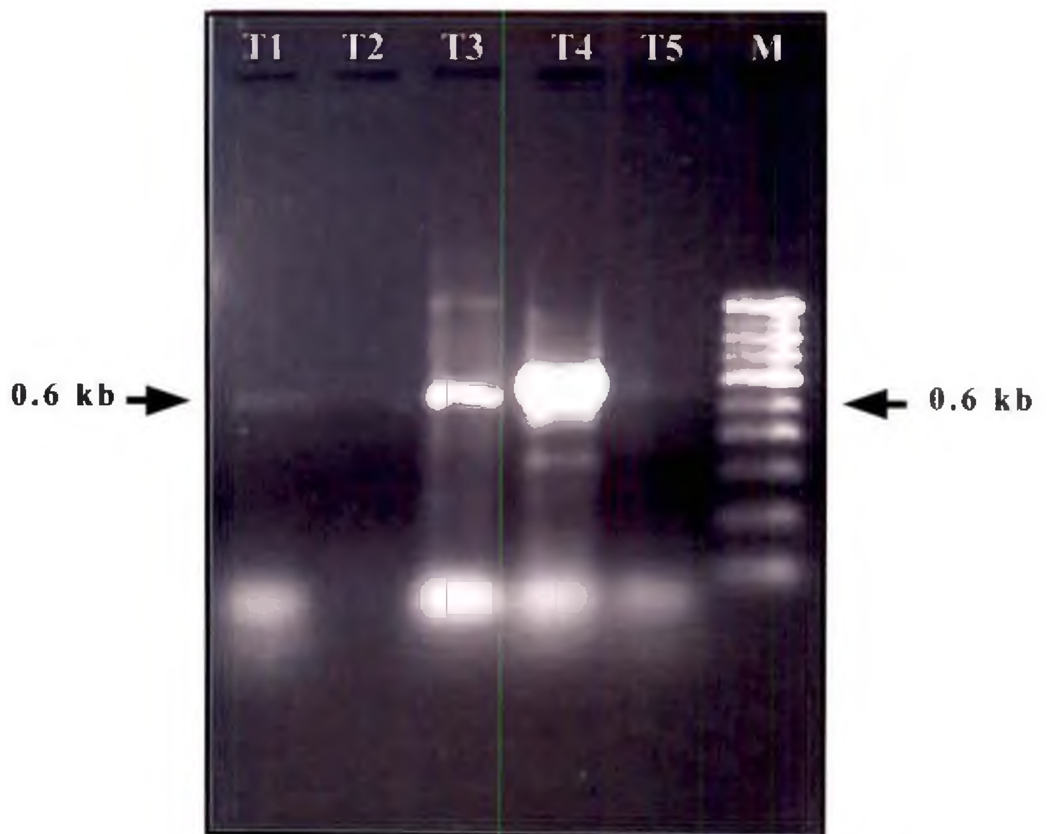

Plate 9. Confirmation of *ACS*, gene in transformants by PCR

Plate 10.

Lane 1- M1- 1 kb DNA marker

Lane 2- P- positive control (*ACS_2* gene)

Lane 3- T1- probe binding band of transformed plant DNA

Lane 4- T2- - probe binding band of transformed plant DNA

Lane 5- T3- non- transformed control

Lane 6- M2- 100 bp DNA marker

Southern Blot Analysis

Southern hybridization was done for PCR confirmed samples. The probe binding pattern of the PCR positive samples were tested with respect to the non transformed plants along with positive control (Plate 10). The probe binding band had the same size as that of the positive control.

Plate 10.

Lane 1- M1- 1 kb DNA marker

Lane 2- P- positive control (*ACS_2* gene)

Lane 3- T1- probe binding band of transformed plant DNA

Lane 4- T2- - probe binding band of transformed plant DNA

Lane 5- T3- non- transformed control

Lane 6- M2- 100 bp DNA marker

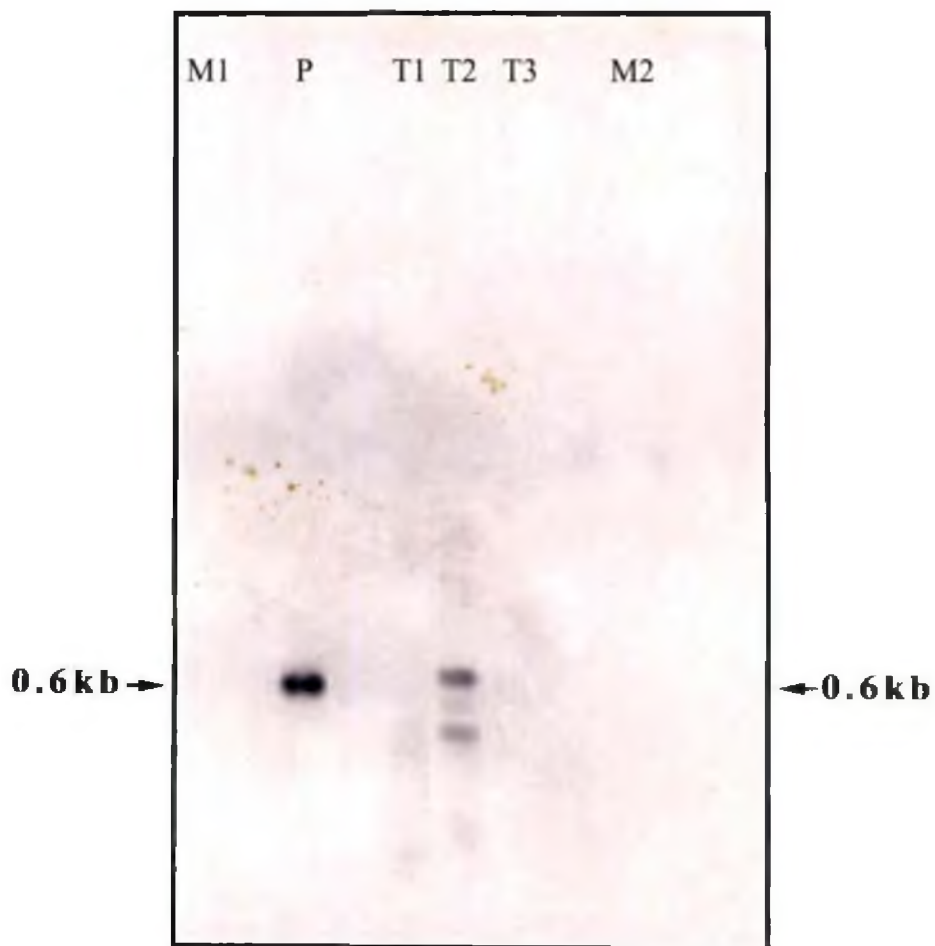

Plate 10. Confirmation of transformation by Southern hybridization

Discussion

5. DISCUSSION

Orchids are one of the important cut flower crops. The Global demand for orchids is on the increase. *Dendrobium* is the popular orchid grown in Kerala. Vase life is an important characteristic determining the commercial value of cut flowers. Several methods are adopted for improving the vase life, viz., regulating the ethylene synthesis pathway, ethylene action, cytokinin production and ABA synthesis. In the present study regulation of ethylene synthesis pathway was aimed at for the improvement of vase life of *Dendrobium* Sonia 17 using antisense gene technology involving 1-amino cyclopropane1- carboxylic acid (ACC) synthase gene. ACC synthase is the key enzyme involved in the synthesis of ethylene. Thus checking the production of ACC synthase using antisense gene technology can block the ethylene production and improve the vase life.

The *Agrobacterium* strain GV3101 harbouring the vector pA_4A_2AB with the ACC synthase antisense gene was tried for improving the vase life. The *in vitro* generation of protocorm like bodies (PLBs) from the meristematic shoot tips required for the study was made possible by fine tuning the protocol (Swarnapiria. 2004) already standardized. The results obtained are discussed below.

The proliferation of PLBs from meristematic shoot tip was the highest when half MS media supplemented with BA 0.2 mg l^{-1} was used.

The *Agrobacterium tumefaciens* strain GV3101 was used for standardization of transformation. The vector, pA_4A_2AB contained the ACC synthase antisense gene (*ACS_2*) and kanamycin resistance gene (*nptII*) as the selectable marker. Plant cells are sensitive to certain antibiotics and they affect regenerative capacity of the explants. Since the genes encoding resistance to antibiotics are used as selectable marker, the sensitivity of tissues to different doses of antibiotics needs to be evaluated. The most effective selection agents are those which either inhibit growth or slowly kill the untransformed tissues.

Bleaching, discolouration and death of callus were observed above 150 mg l^{-1} of kanamycin from the fourth week of treatment. The survival percent of callus in kanamycin 150 mg l^{-1} after eight weeks was 25.60 per cent and this dose was selected for screening the transformants. Kanamycin is a widely used marker for plant transformation and has been used by several workers. Nagaraju *et al.* (1998) reported the use of kanamycin at strength of 100 to 200 mg l^{-1} for selection of *Dendrobium* transformants. Yang *et al.* (1999) observed in *Cymbidium* that all the PLBs cultured on kanamycin 100 mg l^{-1} died 45 days after the subculture. These reports indicated that there is variation in the sensitivity to antibiotic depending on the genotype, physiological condition, size and type of explants and the tissue culture conditions.

Elimination of bacteria from the plant tissues after co-cultivation is very essential, as the bacteria may affect the growth and regeneration of transformed tissues. Commonly used bacteriostatic agent, cefotaxime was used for the elimination of *Agrobacterium* in the present study. At a concentration of 25 mg l^{-1} complete elimination of the bacteria was observed without affecting the growth of the callus. Cefotaxime has been used successfully for the elimination of *Agrobacterium* in transformation in a number of crops. It was successfully used at a strength 150 mg l^{-1} in eliminating *Agrobacterium* harbouring the vector pCAMBIA 1305.1 from the explants in *Dendrobium primulinum.* The PLBs were treated with 250 mg l^{-1} of cefotaxime for the elimination of *Agrobacterium* strain AGL1 containing the vector pCAMBIA1301 in *Dendrobium nobile* (Men *et al.*, 2004). Belarmino and Mii (2000) reported that the elimination of *Agrobacterium* strain LBA4404 strain (pTOK233) could be done by using 300 mg l^{-1} of cefotaxime. The above works indicated that the concentration of cefotaxime required to kill the bacteria depends upon the strain of *Agrobacerium* and the vector that it harbours.

The genetic transformation was done using PLBs of *Dendrobium* Sonia 17. PLBs contain more number of actively dividing cells. These cells are

competent for transformation as reported by Braun (1975). The greater the number of actively dividing cells in the explants to be infected with *Agrobacterium*, the higher was the probability of obtaining stable expression of the transformed gene.

Experiments were undertaken to standardize the optimum time required for the *Agrobacterium* to infect the plant cell. Among the different treatments, infection time of 20 min gave the maximum percentage of putative transformants (27.5) in PLBs. The infection time varied according to the plant species and the bacterial strains. In Gerbera 5 minutes (Nagaraju *et al.*, 1998) and in *Cymbidium* 1.5 hrs (Chan *et al.*, 2003) of infection time resulted in transformation. Sjahril *et al.* (2006) reported that the highest transformation efficiency in *Phalaenopsis* was obtained when the explants were infected for 2 hrs with the *A. tumefaciens* strain EHA101 (pIG121Hm).

Certain polyphenolic compounds like acetosyringone released by the tissue on wounding is necessary for the activation of *Agrobacterium vir* genes. When the *vir* genes were activated, they facilitated the transfer of T-DNA to the plant cell. The quantity of acetosyringone released by the wounded cultured cells may not be sufficient to activate the *Agrobacterium vir* genes. Hence acetosyringone is added to the infection and co-cultivation medium to increase the transformation efficiency. In the present study, addition of acetosyringone 200 µM in the infection and co-cultivation medium increased the transformation efficiency. Belarmino and Mii. (2000) found that acetosyringone 100 µM when added to co-cultivation medium helped in stabilizing the rate of transformation in *Phalaenopsis,* which varied considerably in its absence. Kuehnle and Sugii *et al.* (1992) reported the use of 200 µM acetosyringone to enhance the frequency of transformation in *Phalaenopsis.*

For transformation to be efficient, the induced *Agrobacterium* should have access to cells that are competent for transformation. Hence, the number of days required for co-cultivation was standardized. The transformation efficiency

increased from 22.50 per cent to 27.50 per cent when the co-cultivated period was increased from three to four days. When co-cultivated for more than four days, overgrowth of bacteria was observed. Nebauer *et al.* (2000) obtained optimal transformation rates in *Lavandula latifolia* when leaf explants pre-cultured for one day on regeneration medium were sub cultured on selection medium after a 24 h co-cultivation with *Agrobacterium.* Yu *et al.* (2001) reported that the highest transformation efficiency in *Dendrobium* was obtained when the explants were co-cultivated for three days.

Cultures, after treating with cefotaxime were transferred to selection medium containing kanamycin 200 mg l^{-1}. After two weeks, the non transformed cells turned brown, while the putative transformants remained green. Slightly higher or lower concentration of kanamycin leads to loss of transgenic plants. 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 $l^{-1}$of MS kanamycin sulphate. Yang *et al.* (1999) observed that the transgenic tissues of *Cymbidium* remained green in color in a medium of half strength MS with 150-200 mg $l^{-1}$of kanamycin monosulphate.

The transformed plants showed reduced growth compared to non-transformed plants in selective medium. Kuehle and Sugii (1992) and Belarmino and Mii (2000) also observed that transformed plants show slow growth compared to untransformed ones. Kanamycin concentration that prevented the 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 found adequate for *Dendrobium* by Nan and Kuehnle (1995). Kanamycin at a concentration of 100 µg (Hsieh *et al.* 1997) and 100 mg l^{-1} (Babu and Chawla, 2000) was used to select the transformed plants of *Phalaenopsis* and *Gladiolus*, respectively.

The PLBs grown in high antibiotic stress (200 mg l^{-1}) were selected and DNA was isolated. DNA samples were amplified by PCR with primers specific for the *npt*II and ACS_2 gene. The samples showed a varied quantity of amplified product when the products were run on a one per cent agarose gel. In the case of *npt*II gene only two out of three samples taken for amplification with primer specific for kanamycin resistance gene gave positive result at a distinct band of 0.7 kb. The amplification product of ACS_2 gene was such that out of the four samples, two of them gave appreciable quantity of the product at 0.6 kb. As the initial concentration of the DNA sample used for PCR has been same, this difference in amplification could be attributed to the increased number of integration of the trangene in the genomic DNA of the plant. Nan and Kuehnle (1995) also observed a dosage effect that may arise from gene duplication and multiple insertions in a varied quantity of amplified product for 0.7 kb *npt*II gene fragment. The same result was observed in the PCR analysis of petunia (Shaw et al., 2004) and chrysanthemum (Seo et al., 2003).

The genomic DNA of the PCR positive plants that produced a comparatively higher concentration of amplified product was taken for checking the integration of the transgene. The sample was digested with *Xba*I and *Sac*I and resultant product was hybridized with labeled p-dCTP ACS_2 probe. The sample had the probe bound at the two distinct positions. The band that hybridize to the radio labeled probe represent a copy of transgene as there is a single *Xba*I and *Sac*I sites on either side of the transgene. The variation in the band intensity highlights the difference in the copy number at the site of integration. This confirmatory test has given the proof for the integration of transgene in the plant genome. Thus the bacterial contamination and unstable presence of the transgene in the plant cell can be over ruled.

Molecular breeding of *Dendrobium* is very exciting due to the wealth of new orchid varieties that may be created. During this study various steps for genetic transformation in *Dendrobium* were optimized. The co cultivation time,

infection time, acetosyringone concentration, selection agents, confirmation by PCR and Southern hybridisation were optimized. Based on the results of the study several areas are identified requiring particular attention. The transgenic tissues exhibited a very slow growth rate also, the assessment of sensitivity to antibiotics required more time of about 6 weeks as compared to other crops. However, further research in these fields can improve *Dendrobium* transformation.

Summary

6. SUMMARY

A study on "*Agrobacterium tumefaciens* mediated genetic transformation in *Dendrobium* Sonia 17 with 1- aminocyclopropane-1 carboxylic acid (ACC) synthase antisense gene" was conducted at the Department of Plant Biotechnology, College of Agriculture, and Vellayani during 2004-2006. The salient features of the above study are summarized in this chapter.

The PLBs established *in vitro* derived from meristematic shoot tips were used for the experiment. Surface sterilization of shoot tips explants with mercuric chloride 0.08 per cent for five minutes was found to be very effective. Effect of different doses of coconut water on germination and number of days taken for protocorm initiation was studied. Out of the treatments 150 ml l^{-1} proved to be the best in terms of 88.80 per cent germination and early initiation of protocorm.

Protocol for generation of PLBs was standardized. Among the various treatments tried, half strength MS medium supplemented with BA 0.2 mg l^{-1} was proved to be the best in terms of induction of PLBs with a percentage of 92.50 from the meristematic shoot tip in 25.50 days.

The *Agrobacterium* strain GV3101 harbouring the plasmid pA_4A_2AB was used for genetic transformation. As the plasmid harbors *nptII* as the marker gene, kanamycin was used for the selection of transformants. Hence, experiments were conducted to evaluate the sensitivity of *Agrobacterium* strain GV3101 and PLBs to different concentrations of kanamycin. Bleaching of PLBs was noticed with kanamycin 100 mg l^{-1} from the sixth week onwards. It was observed that the lethal dose of kanamycin to *Agrobacterium* and PLBs were 300 and 150 mg l^{-1}, respectively. For the selection of transformed cells, kanamycin 200 mg l^{-1} was used.

For elimination of *Agrobacterium* after co-cultivation cefotaxime was used. Hence, experiments were conducted to evaluate the sensitivity of *Agrobacterium* and PLBs to different doses of cefotaxime. It was observed that the *Agrobacterium* was effectively killed by cefotaxime 25 mg l^{-1}.

Agrobacterium mediated genetic transformation was achieved by infecting PLBs with bacterial suspension. While optimizing the time required for infection, it was found that as the infection time increased from 15 to 20 minutes the percentage of transformants obtained also increased from 22.50 to 27.50.

Transformation efficiency of 23.50 per cent was obtained when callus was co-cultivated with GV3101 (pA_4A_2AB) for three days. Maximum transformation efficiency of 28.50 per cent was obtained when PLBs was co-cultivated for four days. After co-cultivation the explants were transferred to a medium containing cefotaxime 50 mg l^{-1} to kill the *Agrobacterium*.

The co-cultivated tissues were then transferred to selection medium containing kanamycin 200 mg l^{-1} to select the putative transformants. Survival percentages of PLBs in selection medium, using the strain GV3101 (pA_4A_2AB), was 76.47. The survival per cent of transformants at a reduced kanamycin pressure of 150 mg l^{-1} was 88.23. The transformation efficiency was increased when acetosyringone 200 μM was added to infection and co-cultivation media.

Successful transformation was confirmed by PCR analysis. Out of the kanamycin resistant callus, 66.6 per cent showed PCR positive result. Later the putative transformants were transferred to regeneration medium. The PCR positive transformants were analysed using Southern hybridisation and they gave positive results with the image showing the successful integration on the genomic DNA of the transgenic plant at the same position as the positive control.

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During this study various parameters of genetic transformation in orchid like infection time, co-cultivation time, antibiotic concentration, selection agent, PCR cycles for the amplification of the transgene and Southern hybridisation protocol were optimized. The *Agrobacterium* strain GV3101 (pA_4A_2AB) was efficient for transformation in *Dendrobium* Sonia 17. This study provides a protocol for genetic transformation in *Dendrobium* Sonia 17 using ACC synthase antisense gene for improved vase life.

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* Originals not seen

Appendices

APPENDIX I

Chemical composition of media employed for the *in vitro* culture of *Dendrobium* Sonia 17

MS medium

(i) Macro-nutrients (mg l^{-1})

$Mg SO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170

(ii) Micro-nutrients (mg l^{-1})

$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$AlCl_3$	0.025
KI	0.83
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25

(iii) Iron Source (mg l^{-1})

$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25

(iv) Vitamins (mg l^{-1})

Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

(v) Amino acid source (mg l^{-1})

Glycine	2.0
Inositol (mg l^{-1})	100
Sucrose (g l^{-1})	30
Agar (g l^{-1})	6

APPENDIX II

Chemical composition of media employed for the culture of *Agrobacterium tumefaciens*

Yeast Extract Mannitol Medium (YEM Medium) (g l^{-1})

Yeast Extract	0.4
Mannitol	10.0
NaCl	0.1
MgSO4. 7 H2O	0.2
K2HPO4	0.5
Agar	15.0
p^H	7

AB Minimal Medium

AB salts (g l^{-1}) 20 X stock

NH_4Cl	20.0
$MgSO_4.7H_2O$	25.0
KCl	3.0
$CaCl_2$	0.2
$FeSO_4.7H_2O$	0.05
p^H	7

AB buffer (g l^{-1}) 20 X stock

K_2HPO_4	60.0
NaH_2PO_4	23.0

Glucose	0.5%
Agar	1.5%

Working solution 1X

APPENDIX III

Chemicals for isolation of DNA from *Dendrobium* Sonia 17

CTAB Buffer

CTAB - 2%

Tris- HCl, pH- 8.0 - 100mM

NaCl - 1.4 M

EDTA - 20mM

2- mercapto ethanol - 0.1%w/v

TE BUFFER pH 8.0

Tris buffer - 10mM

EDTA - 1mM

50 X TAE BUFFER pH 8.0

Tris Buffer - 24.20g

Glacial acetic acid - 5.71 ml

EDTA - 0.05 M

APPENDIX IV

Chemicals for isolation of plasmid DNA from *Agrobacterium* strain GV3101

(i) Solution I

20% glucose - 2.25 ml

0.5M EDTA (pH- 8.0) - 1 ml

1M Tris (pH- 8.0) - 1.25 ml

Sterile distilled water - 45.50 ml

(ii) Solution II

10 N NaOH - 0.4 ml

20% SDS - 1 ml

Sterile distilled water - 18.6 ml

(iii) Solution III

5 M Sodium acetate - 60 ml

Glacial acetic acid - 11.5 ml

Sterile distilled water - 28.5 ml

APPENDIX V

Chemicals for Southern hybridisation of the transformants

(i) Depurination solution

Conc. HCl - 20.8 ml

Sterile distilled water- 1 litre

(ii) Denaturing Solution

NaOH - 20 g (0.5 M)

NaCl – 29.2 g (1.5 M)

Sterile distilled water- 1 litre

(iii) Neutralization Solution (pH- 5.0)

Tris Cl – (1.5 M) pH- 7.2

NaCl- 2.0 M

EDTA- 1 mM

(iv) SSC Solution (pH- 7.0) – 20X

NaCl- 175.32g (3 M)

Tri sodium citrate dehydrate- 88.23 (300 mM)

Sterile distilled water- 1 litre

(v) Pre- hybridisation Buffer

SDS- 5.6 g (7%)

Phosphate buffer- 1 M (20 ml)

EDTA- 0.5 M (1.6ml)

BSA- 1% (20ml)

Sterile distilled water- 40 ml

(vi) Hybridisation Buffer

Pre- hybridisation Buffer

Denatured labeled probe

(vii) Washing Buffer

Washing solution 1

2X SSC + 0.1% SDS

Washing solution 2

1X SSC + 0.1% SDS

Washing solution 3

0.2X SSC + 0.1% SDS

APPENDIX VI

Nucleotide sequence and structure of the vector, pA_4A_2AB in GV3101

The sequence of the LeACS4 promoter which is driving the expression of the LeACS2 cDNA in the construct.

```
LOCUS       AF548375                      414 bp    DNA     linear
PLN 01-OCT-2003
DEFINITION  Lycopersicon esculentum ACS4 gene, partial sequence.
ACCESSION   AF548375
VERSION     AF548375.1  GI:33334357
KEYWORDS    .
SOURCE      Lycopersicon esculentum (Solanum lycopersicum)
ORGANISM  Lycopersicon esculentum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
eudicotyledons;
            asterids; lamiids; Solanales; Solanaceae; Solanum;
Lycopersicon.
REFERENCE   1  (bases 1 to 414)
  AUTHORS   Anjanasree,K.N. and Bansal,K.C.
  TITLE     5' sequence of ripening-regulated LeACS4 gene of
tomato
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 414)
  AUTHORS   Anjanasree,K.N. and Bansal,K.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-SEP-2002) National Research Center on
Plant
            Biotechnology, Indian Agricultural Research Institute,
IARI Campus,
            New Delhi 110012, India
FEATURES             Location/Qualifiers
     source          1..414
                     /organism="Lycopersicon esculentum"
                     /mol_type="genomic DNA"
                     /cultivar="Pusa Ruby"
                     /db_xref="taxon:4081"
```

```
gene            1..>414
                     /gene="ACS4"
                     /note="LeACS4; coding region not determined"
ORIGIN
        1 aatttgaata tgttttaact ttttaaatat tttgacgtaa gtttttttgtt
ttcccccaaa
       61 agtttcacat tagactaaat ttaatttcct ataacttggt ttctaatatt
tttgacgggt
      121 tgaaagcgtc ataaatgact aaatcttttt cttaaaagag cgtaaattct
gccttgtttt
      181 aatctcagtc ttccactttt ttcatgccat aaaagattaa aatctttttc
ttggaggagc
      241 gtagattttg actttatttt aatcccaatt tcttactctt tcgatgaggc
aagcataacc
      301 tcaccacact acactacact tttggacaca aagcatttct tcaattctcc
ttagctataa
      361 aaacccctca tagcttcttc attctcttcc aaacaaaaac ttttgtactt
caaa

//
```

The sequence of the LeACS2 cDNA in the vector

```
LOCUS       AY326958                1459 bp    mRNA    linear
PLN 01-JUL-2004
DEFINITION  Lycopersicon esculentum ripening-related ACC synthase
2 (ACS2)
            mRNA, complete cds.
ACCESSION   AY326958
VERSION     AY326958.1  GI:33149674
KEYWORDS    .
SOURCE      Lycopersicon esculentum (Solanum lycopersicum)
  ORGANISM  Lycopersicon esculentum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
eudicotyledons;
            asterids; lamiids; Solanales; Solanaceae; Solanum;
Lycopersicon.
REFERENCE   1  (bases 1 to 1459)
  AUTHORS   Anjanasree,K.N. and Bansal,K.C.
  TITLE     Cloning of ripening-related ACC synthase 2 (ACS2) cDNA
from tomato
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1459)
  AUTHORS   Anjanasree,K.N. and Bansal,K.C.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-JUN-2003) National Research Center on
Plant
            Biotechnology, Indian Agricultural Research Institute,
Pusa Campus,
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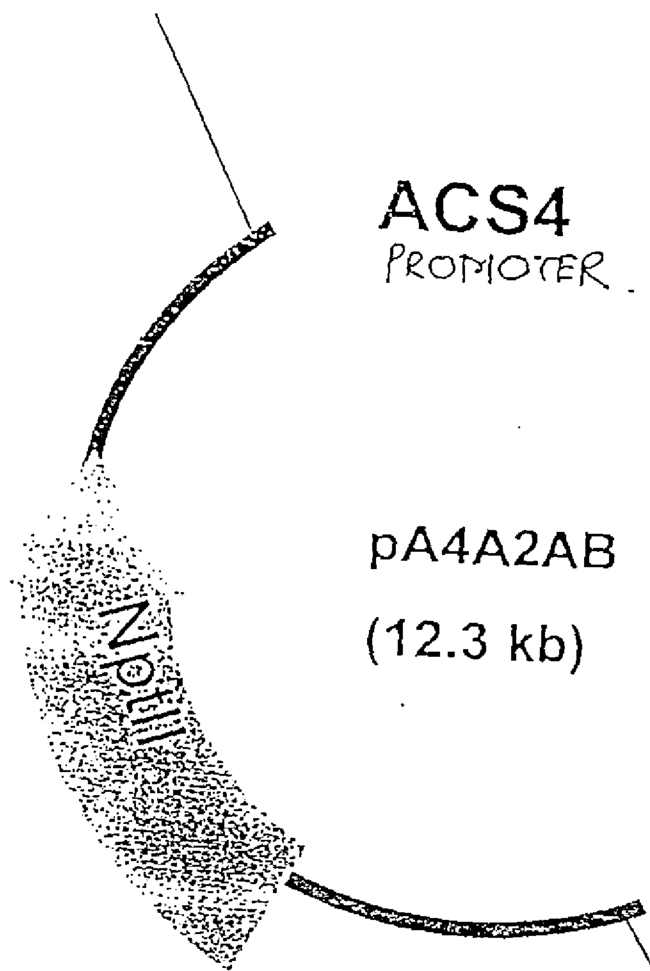
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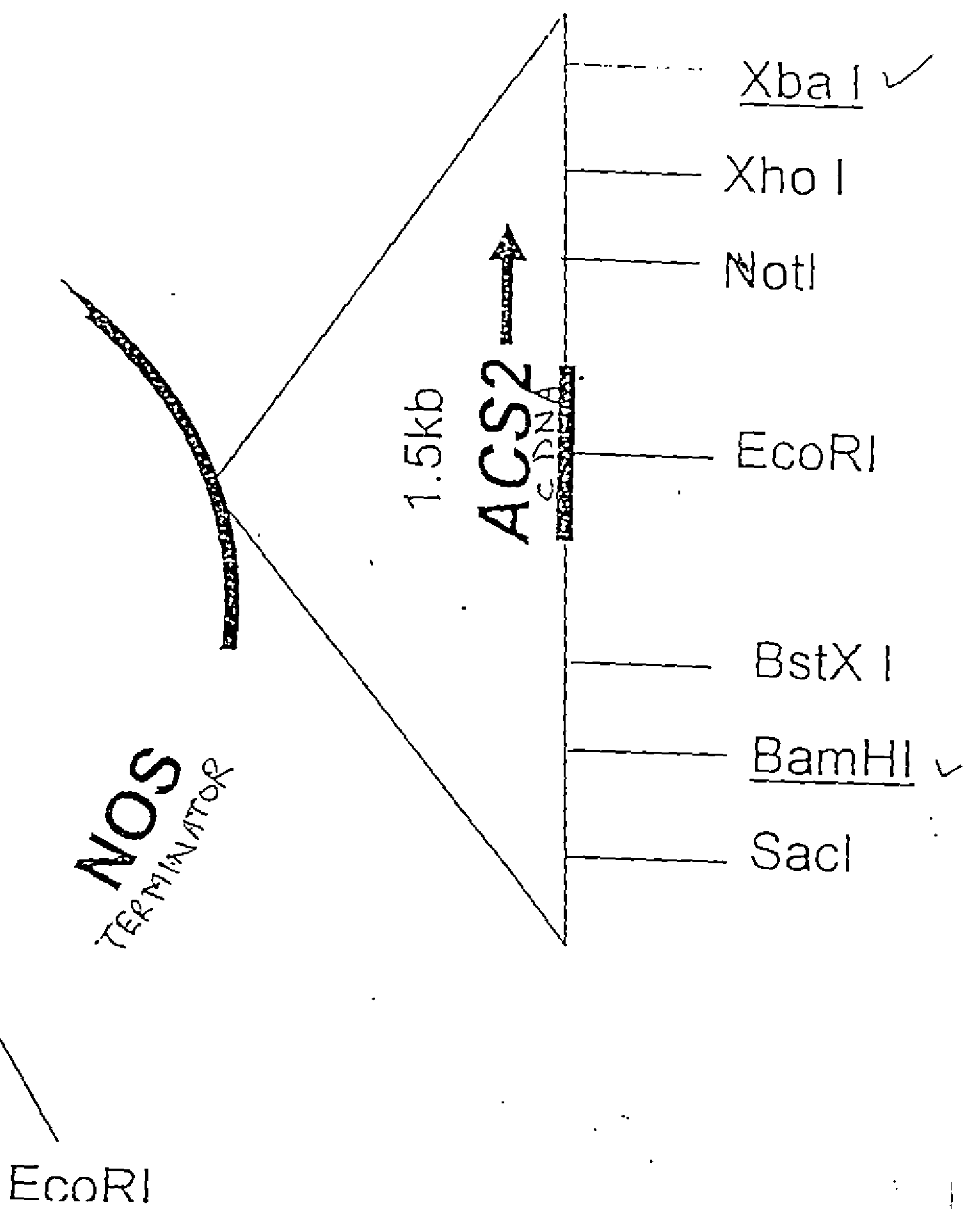
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AGROBACTERIUM TUMEFACIENS MEDIATED GENETIC TRANSFORMATION IN *DENDROBIUM* VARIETY SONIA 17 WITH 1- AMINOCYCLOPROPANE- 1 CARBOXYLIC ACID (ACC) SYNTHASE ANTISENSE GENE

KARTHIKA KARUNAKARAN

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

Master of Science in Agriculture

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Department of Plant Biotechnology
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522

ABSTRACT

A study on "*Agrobacterium tumefaciens* mediated genetic transformation in *Dendrobium* variety Sonia 17 with 1- aminocyclopropane-1 carboxylic acid (ACC) synthase antisense gene" was conducted at the Department of Plant Biotechnology, College of Agriculture, and Vellayani during 2004-2006.

Orchid is an important cut flower crop. *Dendrobium* Sonia 17 is the most popular commercial orchid grown in Kerala. It has sufficiently higher vase life. However, increasing vase life can reduce the per day cost of flower. The present study was undertaken with the main objective of evolving a protocol for *Agrobacterium tumefaciens* mediated genetic transformation in *Dendrobium* Sonia 17 using ACC synthase antisense gene.

PLBs were initiated from the meristematic shoot tip on half strength MS medium supplemented with growth regulators. MS medium supplemented with BA 0.2 mg l^{-1} was proved to be the best in terms of induction of PLBs (92.5 %).

The *Agrobacterium tumefaciens* strain GV3101, harbouring the plasmid pA_4A_2AB was used for genetic transformation. As the plasmid harbour *nptII* and ACS_2 genes, the sensitivity of *Agrobacterium* strain and PLBs to different concentrations of kanamycin was evaluated. The lethal doses of kanamycin to *Agrobacterium* and PLBs were 300 and 150 mg l^{-1}, respectively. The effective dose of cefotaxime for the elimination of bacterial strain GV3101 was 50 mg l^{-1} and the lethal dose of cefotaxime to PLBs was 300 mg l^{-1}.

Genetic transformation was achieved by co-cultivating PLBs with bacterial suspension. Conditions like infection and co-cultivation time, selection agent were optimized. The most effective infection time was 20 min, followed by a co-cultivation period of four days. The survival of tissues transformed on the selection media was

76.47 per cent. The transformation efficiency was increased when acetosyringone 200 μM was added to infection and co-cultivation media.

Transformation was confirmed by PCR and southern hybridisation of putative transformants. This study provides a protocol for genetic transformation in *Dendrobium* sonia 17 using ACC synthase antisense gene.

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