MANAGEMENT OF RECALCITRANCY IN *IN VITRO* CULTURES OF CASHEW (*Anacardium occidentale* L.)

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Management of recalcitrancy in *in vitro* cultures of cashew (*Anacardium occidentale* L.)"** is a bonafide record of research work done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Management of recalcitrancy of in *in vitro* cultures of cashew (*Anacardium occidentale* L.)"** is a bonafide record of research work done independently by **Ms. Jusna Mariya P.L.** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Jusna Mariya P.L.

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ABBREVIATIONS

AC	Activated charcoal
A. rhizogenes	*Agrobacterium rhizogenes*
AMF	Arbuscular Mycorrhizal fungi
ATCC	American Type Culture Collection
BA	Benzyl adenine
BAP	Benzyl amino purine
BIM	Bacteriological indexing medium
BLAST	Basic Local Alignment Search Tool
bp	Base pair
B5	Gamborg B5 medium
Ca	Calcium
$CaCl_2$	Calcium chloride
Cd	Cadmium
cm	Centimeter
CO_2	Carbondioxide
CPBMB	Centre for Plant Biotechnology and Molecular Biology
Cu	Copper
CW	Coconut water
^0C	Degree Celsius
Da	Dalton
DAT	Days After Transplanting
DICA	Direct Inoculation and Co-cultivation with Acetosyringone
DIM	Direct Inoculation Method
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
ds	Double stranded
EDTA	Ethylene Diamine Tetra Acetic acid
Fe	Iron

γ	Gamma
g	Gram
gl^{-1}	Gram per litre
GA_3	Gibberellic acid
ha	Hectare
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HPLC	High Performance Liquid Chromatography
hr	Hour (s)
2iP	Iso pentyl adenine
IAA	Indole acetic acid
IBA	Indole butyric acid
IMTECH	Institute of Microbial Technology, Chandigarh
IPTG	Isopropyl thio galactoside
K	Potassium
KAU	Kerala Agricultural University
kb	Kilo base pair
Kg	Kilogram
Kin	Kinetin
KOH	Potassium Hydroxide
L	Litre
LB	Luria Bertani
LBA	Luria Bertani Agar
LS	Linsmaier and Skoog medium
m	Meter
M	Molar
Mg	Magnesium
mgl^{-1}	Milli gram per litre
$MgCl_2$	Magnesium chloride
min	Minute(s)
ml	Milli litre

mm	Milli meter
mM	Milli Molar
MS	Murashige and Skoog's medium
MTCC	Microbial Type Culture Collection
µg	Micro gram
µl	Micro litre
µM	Micro Molar
M	Molar
N	Nitrogen
Ni	Nickel
NA	Nutrient Agar
NAA	Napthalene acetic acid
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nano gram
nm	Nano meter
OD	Optical density
ORF	Open Reading Frame
P	Phosphorus
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pH	Hydrogen ion concentration
ppm	Parts per million
psi	Pound per square inch
PVP	Polyvinylpyrrolidone
%	Percentage
RH	Relative Humidity
rDNA	Ribosomal Deoxyribonucleic Acid

rpm	revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
SCA	Suspension culture and Co-cultivation with Acetosyringone
SDS	Sodium Dodecyl Sulphate
sec	Second (s)
SH	Schenk and Hildebrandt medium
SM	Suspension culture inoculation Method
TAE	Tris Acetate EDTA
TDZ	Thidiazuron
T-DNA	Transfer DNA
TIBA	Tri iodo benzoic acid
TL-DNA	Transfer (left) DNA
TR-DNA	Transfer (right) DNA
SDS	Sodium Dodecyl Sulphate
U	Unit
UV	Ultra Violet
V	Volts
VAM	Vesicular Arbuscular Mycorrhizae
WPM	Woody Plant Medium
w/v	Weight by volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase
YEB	Yeast Extract Broth
YEM	Yeast Extract Mannitol
Zn	Zinc

Dedicated to

My loving

Parents

&

Brothers

Introduction

INTRODUCTION

Cashew (*Anacardium occidentale* L.) is the most promising crop of neglected and barren lands and a foreign exchange earner in several developing countries of tropical regions. The plant belongs to the genus *Anacardium*, a member of family *Anacardiaceae* (Nakasone and Paull, 1998). Behrens (1998) described cashew as a tropical tree species cultivated in many tropical countries of the world and which has spread from its centre of origin in south and central America to Africa, Asia and Tropical Australia. The crop was introduced to India, Asia and Africa in the 15th and 16th centuries (Woodroof, 1967; Ohler, 1979).

The major cashew producing countries are Tanzania, India, Mozambique, Sri Lanka, Kenya, Madagascar, Thailand, Malaysia, Indonesia, Nigeria, Senegal, Malawi and Angola. India is the largest producer, exporter and consumer of cashew in the world. Today, cashew has become a crop of high economy and has moved from a crop of waste lands to a commercial crop (Singh, 1998). During 2007-08, export of cashew kernels amounted to 1,14,340 million tonnes earning Rs.2,28,890 lakhs of foreign exchange, export of cashewnut shell liquid amounted to 7,813 million tonnes earning Rs.1,197 lakhs of foreign exchange and export of raw nuts amounted to 6,05,970 million tonnes earning Rs.1,74,680 lakhs of foreign exchange (Directorate of Cashew and Cocoa Development, 2008). Cashew industry provides employment (direct and indirect) to about 10 lakh workers, 95 per cent of whom are rural women from the underprivileged sections of the society.

Average productivity of cashew in India during the year 2007-08 was reported to be 860 Kg/hectare (Directorate of Cashew and Cocoa Development, 2008). But the potential of this crop is 1777 Kg/ hectare. One of the

reason for the low productivity is the presence of senile and low productive plants. Maximum yield potential can be achieved through replanting or replacing the low productive and unproductive cashew plantations in the different states. Non-availability of elite planting material in adequate number is the main constraint in this regard.

Cashew is highly cross-pollinated, woody and perennial in nature and the progress made in improvement of this crop is slow. It is propagated mainly by seeds and this often results in high levels of variability (Phlip and Unni, 1984). A faster clonal multiplication technique like micropropagation would be a useful supplement to the conventional vegetative propagation methods, e.g. air layering and grafting which are not sufficient to produce plants as much as needed. But cashew, like other *Anacardiaceae*, is strongly recalcitrant to *in vitro* culture and only limited successes have been achieved as yet: direct shoot and root regeneration was obtained from proximal ends of cotyledons (Philip, 1984; Philip and Unni; 1984) and calli of different explant origin have been reported to produce roots (Leva and Falcone, 1990; Sy *et al*., 1991) or globular protuberances which developed into embryo like structures but with many aberrations (Jha, 1988). Progress with application of micropropagation of cashew has been achieved using microshoots (Lievens *et al*., 1989; Leva and Falcone, 1990) and cotyledonary nodes (D'Silva and D'Souza, 1992a; Keshavachandran, 1996; Das *et al*., 1996), shoot nodal explants from glass house raised plants (Boggetti *et al*., 1999), embryo axis (Ananthakrishnan *et al*., 1999), shoot tip and node/axillary buds (Keshavachandran and Shelja, 1999; Thimmappaiah and Shirly, 1999; Keshavachandran, 2004). Induction of somatic embryogenesis from mature and immature cotyledon sections (Hedge *et al*., 1990, 1991), nucellar-derived callus of cashew (Ananthakrishnan *et al*., 1999; Gogate and Nadgauda, 2003) and embryo axis (Aliyu and Awopetu, 2005) has also been reported.

However development of a successful, consistent *in vitro* multiplication protocol is a problem in cashew due to various reasons. Hence in order to assess and manage the recalcitrance of *in vitro* propagation in cashew, the present study was taken up with the following objectives.

1. To assess and manage the recalcitrance of *in vitro* cultures in cashew with respect to the phenolic exudation

2. To assess and manage the recalcitrance with respect to endophytic microbial contamination

3. Induction of rooting with *Agrobacterium rhizogenes*

4. Improvement of transplantation success by treatment with AMF-PGPR consortium

Review of Literature

2. REVIEW OF LITERATURE

Cashew (*Anacardium occidentale*) is a native of North-east Brazil and its area of origin is reported to have been the state of Ceara where the tree is found to this day in large groves. Its diffusion in Asia and Africa is due to the Portuguese in the first half of the 16th century (Agrolioni and Gailians, 1977).

Cashew is commercially grown for its kernels although cashew nut shell liquid (CNSL) and apple are also valuable by products. It is considered to be the most important edible nut in the world trade (Ascenso, 1986). Cashew crop which has been considered only useful for soil conservation, afforestation and development of waste lands, has today assumed a status of an important dollar earning horticultural crop in India (Chandha, 1991). After the second world war the world production and consumption of cashew increased sharply, and it soon became the world's most important desert nut after almonds (Thimmappaiah *et al.*, 2001).

Propagation through seeds is the natural means of propagation. Since cashew is a cross pollinated crop, there will be a great variability between trees raised from seeds of a single mother tree. Hence, vegetative method of propagation is resorted to get uniformity among the progeny for desirable characters. Though vegetative propagation methods such as air layering and softwood grafting are widely used, the rate of multiplication and field establishment are by no means commensurate with the demand for superior planting materials. Hence, development and standardization of *in vitro* propagation techniques for rapid multiplication of quality planting materials on a large scale will be useful.

Application of tissue culture methods have been recommended for the following aspects of cashew to: (i) supplement the vegetative propagation through production of clonal planting materials by direct shoot bud regeneration,

(ii) generate more scion material for use in grafting, (iii) obtain rooted plants directly from elite trees, (iv) develop an *in vitro* system for screening germplasm, hybrids and other induced variability of resistance/tolerance to diseases and pests and (v) explore the possibility of inducing mutation or somaclonal variations for higher productivity and quality as well as resistance/tolerance to diseases pests and stress environment (Nambiar and Iyer, 1988). Besides use of molecular markers will help in more efficient germplasm management of cashew and in genetic fingerprinting of the improved selections and hybrids that have been evolved in cashew.

2.1 Genus Anacardium

A genus of 11 species, mostly in tropical America. Leaves entire, inflorescence terminal, bracteate. Flowers polygamous, 5-merous, petals reflexed at length, stamens 8-10, unequal, 1-2 perfect, fruit a nut supported by fleshy pseudocarp (Bose *et al.*, 1998).

2.1.1 *Anacardium occidentale* Linn.

An erect, spreading, evergreen tree, growing up to height of 15 m with rough bark. Leaves glabrous, thickly coriaceous, obovate, 10-16 cm long entire. Flowers polygamous, 1-1.5 cm across in large terminal bracteate panicles, pentamerous. Petals linear, pale yellow streaked with pink; stamens 10, one longer and always fertile, the rest of ten sterile. Fruits a drupe, kidney shaped, 2-3 cm long, seated on a fleshy, thick pseudocarp, 5-8 cm long orange or crimson (Bose *et al.*, 1998).

2.1.2 Climate and soil

Cashew is hardy and drought-resistant tropical crop. It grows well from mean sea level to an elevation of 1,000 m. It thrives well in the areas with an annual rainfall ranging from 600 to 4,000 mm and temperature ranging from 15 to 45^0C but a relatively high humidity and temperature between 19^0C and 35^0C are the best condition. It is highly susceptible to frost. Heavy rains during flowering leads to poor fruit set. High temperature during marble stage of fruit development causes fruit drop (Bose *et al*., 2006).

Though cashew can be grown on a variety of soils, a well-drained 1-1.5 m deep soil and pH of 5.6 to 7.0 is preferred. Laterites, red and coastal soils rich in organic matter are best for cashew growing. Even the marginal soils can be used for cashew cultivation but the waterlogged condition should be avoided, as cashew is very susceptible to it (Bose *et al*., 2006).

2.1.3 Cultivated areas

The tree is common in tropical America from Mexico to Peru and Brazil. It was introduced to India from Brazil by the Portuguese some 400 years ago and has become naturalized in the West cost of India. It is also cultivated in the coastal regions of South Africa, Malagassy, Mozambique, West Indies and is found in South-East Asia from Sri Lanka to Philippines.

2.2 Application of *in vitro* plant regeneration in *Anacardium occidentale*

In vitro propagation techniques have applications in root stock propagation where the clonal rootstocks produced will impart uniformity in stand and performance to scion variety in field, for an early induction of a newly released variety due its speedy multiplication of F_1 hybrid/elite clone whose planting material is limited in the initial stage and to take up *in vitro* conservation

of germplasm and regeneration of transgenics. Besides these, use of molecular markers will help in more efficient germplasm management of cashew and in genetic fingerprinting of the improved selections and hybrids that have been evolved in cashew (Thimmappaiah *et al.*, 2001).

2.2.1 Status of *in vitro* propagation in *Anacardium occidentale*

Cashew is strongly recalcitrant to *in vitro* culture and only limited successes have been achieved as yet direct shoot and root regeneration was obtained from proximal ends of cotyledons (Philip, 1984; Philip and Unni, 1984) and calli of different explant origins have been reported to produce roots (Leva and Falcone, 1990; Sy *et al.*, 1991) or globular protuberances, which developed into embryo-like structures but with many aberrations (Jha, 1988). Progress with application of micropropagation has been achieved using micro shoots (Lievens *et al.*, 1989; Leva and Falcone, 1990) and cotyledonary nodes (D'Silva and D'Souza, 1992b; Das *et al.*, 1996) but many problems still persist with *in vitro* explant viability, bud sprouting and shoot elongation. Additionally *in vitro* rooting and survival of *in vitro* produced plants remain constraints to more widespread application of micropropagation techniques to cashew (Leva and Falcone, 1990; D'Silva and D'Souza, 1992a; Das *et al.*, 1996). Boggetti *et al.* (1999) reported that *in vitro* multiplication of cashew using shoot nodal explants from glass-house raised plants. *In vitro* plant regeneration from embryo axis of cashew nut (Ananthakrishnan *et al.*, 1999), shoot tip and nodal axillary buds (Thimmappaiah and Shirly, 1999) have been reported.

Induction of somatic embryogenesis has been achieved from nucellar derived callus of cashew (Ananthakrishnan *et al.*, 1999; Gogate and Nadgauda, 2003). Direct somatic embryogenesis from mature and immature cotyledon sections (Hedge *et al.*, 1991) and embryo axis (Aliyu and Awopetu, 2005).

Micropropagation of cashew from mature trees has not been very successful mainly due to problems of contamination, heavy leaching of phenolics and the recalcitrant nature of the tissues (D'Souza *et al*., 1996). Often slow growth, chlorosis, drying of tissues and rooting inability have also been encountered. Another factor attributing to the poor *in vitro* response from mature tree is the lack of a juvenility factor in older trees, and rejuvenation of such shoots was found to help in regeneration (Pierik, 1990; D'Souza *et al*., 1999).

2.2.1.1 Source of explant

2.2.1.1.1 *In vitro* source

Philip (1984) reported regeneration of explants from fragmented cotyledons of an undefined cultivar. Calli of different explant origins have been reported to produce roots (Leva and Falcone, 1990; Sy *et al*; 1991). Micropropagation of cashew has been achieved through cotyledonary nodes (D'Silva and D'Souza, 1992a; Das *et al*., 1996). Ananthakrishnan *et al*. (2002) reported *in vitro* adventitious shoot formation from cotyledon explants of cashew.

2.2.1.1.2 *Ex vitro* source

Lievens *et al*. (1989) and Leva and Falcone (1990) successfully cultured microshoots derived from 6-15 month and 3 year old plants, respectively. Boggetti *et al*. (1999) reported *in vitro* multiplication of cashew using shoot nodal explants from glass-house raised plants. *In vitro* plant regeneration from embryo axis of cashew nut (Ananthakrishnan *et al*., 1999), shoot tip and nodal axillary buds (Thimmappaiah and Shirly, 1999) were also reported.

2.2.1.2 Surface sterilization of explants

Many workers encountered serious problem of contamination while initiating cultures of tree species (Wilkins *et al.*, 1985). Microbial contamination of plant tissue culture is usually caused by fungi or bacteria which may be epiphytic or endophytic (Litz and Conover, 1981; Leifert *et al.*, 1994). These micro organisms outgrow the explant tissue and cause loss of cultures (De Fossard, 1976).

Although both fungi and bacterial contaminations were observed in cashew, Ramanayake and Kovoor (1997) observed presence of systemic fungus in shoots excised from mature trees. Similarly, fungal contaminants were found to be predominant in mature tree cultures and were identified to be *Fusarium* (Thimmappaiah and Shirly, 1996). In mature seeds, the actual contaminant was also a fungus in addition to bacteria. The pericarp and remnants of the juicy stalk attached to seeds were found to be the source of contaminants (D'Souza *et al.*, 1996). Maintenance of stock plants by periodic sprays with fungicides (Carbendazim, dithane, aureofungin) is advisable before collecting the explants. D'Silva and D'Souza (1993) found reduction in fungal contamination when the explants were agitated in 200 mg l^{-1} of carbendazim (Bavistin) for 5 hours and / or by incorporating in the medium. The best recourse to control contamination in cultures of seedlings is to germinate the seeds *in vitro*.

Sterilization of mature and immature seeds is done by either mercuric chloride (0.1-0.2%) or 0.1 per cent sodium hypochlorite dip. Tender shoots (3-4") collected are defoliated and made into smaller segments (shoot tips and nodal cuttings). After a brief dip in 70 per cent alcohol, the explants are washed thoroughly in sterile distilled water by adding a pinch of detergent and then in 0.1 per cent carbendazim or 0.3 per cent dithane M-45 for 2 min. In laminar flow, sterilization is done by constant agitation in 0.1 per cent mercuric chloride with 1-2 drops of Tween-20 (5-8 min). After rinsing the explants

thoroughly 3-5 times in sterile distilled water, they were segmented to size before inoculating them in proper media. Chlorine and hypochlorite solutions were found to cause discolouration and browning in cashew. Hence mercuric chloride, though toxic, proved useful for the control of contamination (Thimmappaiah, 1997). For shoot tips and leaf segments a lower concentration of mercuric chloride (0.05%) was found useful.

In the work carried out at KAU, Vellanikkara, nuts were harvested from experimental plantings and washed thoroughly with tap water and then with water to which a few drops of Teepol was added. The nuts were then treated with 1 per cent Cetrimide for 45 min and then with 1.5 per cent Emisan for 1 hour before soaking in sterile water for 3 days. The soaked nuts were then treated with 0.1 per cent mercuric chloride for 30 min, dipped in alcohol and flamed prior to the removal of the outer shell. The seed coat was removed and the embryo with the cotyledons inoculated into proper medium (Keshavachandran, 2004).

Shoot tips and nodal segments were taken from grafted plants which were sprayed regularly with Bavistin. The explants were first washed thoroughly in tap water to which a few drops of Teepol had been added. The explants were then treated with a solution of 0.1 per cent Bavistin and a few drops of Teepol and shaken for 45 min followed by soaking in 0.1 per cent mercuric chloride to which 0.1 per cent sodium lauryl sulphate had been added for 5 min. The treated explants were then inoculated into proper medium (Keshavachandran, 2004).

2.2.1.3 Interference due to polyphenols

Browning of explants (necrosis) and media due to exudation of phenolics is one of the serious bottle-necks in the establishment of cultures from

trees (Preece and Compton, 1991). It is found to be extremely severe in cashew as it contains high amounts of phenols (D'Silva and D'Souza, 1993). Among the different explants studied, leaf had the highest content of phenols and orthohydroxy phenols followed by shoot-tips and nodal cuttings (Thimmappaiah, 1997). Among the various methods suggested for controlling contamination are soaking of explants in 0.28 mM ascorbic acid before inoculation (D'Silva, 1991), keeping cultures initially in the dark for 3 weeks (Hedge, 1988; Nair and Mohanakumaran, 1993), incorporating activated charcoal (0.1-1%) in medium (Nair and Mohanakumaran, 1993; Das *et al.*, 1996; Thimmappaiah, 1997) ascorbic acid (Nambiar and Iyer, 1988; Lievens *et al.*, 1989; Thimmappaiah, 1997) and PVPP incorporation in media. Reduction in phenolic exudation was observed when the preparation of explants and culturing was done under low temperature condition and by adding 50 mg l^{-1} activated charcoal, 150 mg l^{-1} ascorbic acid and 200 mg l^{-1} calcium pantothenate into the culture medium (Hedge and Kulasekaram, 1994).

A combination of different methods has also been seen to be useful (Keshavachandran and Khader, 1990; Keshavachandran and Menon, 1997). The browning of the explants were controlled by treating the explants with 1 g l^{-1} PVP, incorporating 0.05 per cent activated charcoal in the medium and keeping the cultures initially in dark for 7 days.

2.2.1.4 *In vitro* seed germination

As cashew is highly prone to contamination, there is a need for raising plants under hygienic condition. For this the seeds which are healthy are selected, dipped in alcohol, flamed and sterilized in 0.1 per cent mercuric chloride or commercial bleach and sown in trays/polybags containing sterile sand and soil mixture and maintained under greenhouse or laboratory condition (growth chamber). To hasten and get uniform germination, the seeds are soaked in 36 per

cent acetone for 2 hours (Lievens *et al.*, 1989; Sy *et al.*, 1991). Alternatively, seedlings may be raised under aseptic condition which is not only free from contaminants but also free from toxic effect of sterilants.

Hedge *et al.* (1991) germinated seeds from 5 to 7 week old nuts on MS (Murashige and Skoog, 1962) and LS (Lin and Staba, 1961) media containing varying levels of auxins and cytokinins. The nuts were thorough by washed in running water and in distilled water (2-3 times) and sterilized in 2 per cent sodium hypochlorite (30 min) followed by 3-4 times rinsing with sterile distilled water. Holding the nut with the help of forceps, an incision of 2-3 cm into the shell was made. The shell was removed, and the whole kernel with intact embryo was scooped out. Embryo with part of the cotyledon was embedded in the medium. By 6 weeks, 8-10 cm long seedlings were obtained. MS medium containing 2 mg l^{-1} NAA resulted in 100 per cent seed germination.

D'Silva and D'Souza (1993) obtained *in vitro* germination of mature nuts by culturing seeds on plain agar medium. After thorough washing of mature nuts in distilled water, surface-sterilization was done in 0.1 per cent mercuric chloride and 0.1 per cent sodium laural sulphate for 15 min. With several rinses in sterile distilled water, they were germinated in screw cap bottles containing 70 ml of 0.6 per cent plain agar medium. Another batch was germinated by soaking one seed each in a bottle containing sterile distilled water (30 ml/bottle) for 8 days. They were dissected out in laminar flow to remove the seed. The seeds after peeling of the testa were germinated in plain agar medium as above. Similarly, immature nuts were sterilized for 15 min in 0.1 per cent mercuric chloride followed by twice rinsing in sterile distilled water. The pericarp was cut open in laminar flow, and the seed was lifted out. After careful removal of the seed coat, the decoated seeds were germinated in plain agar medium.

For germinating cashew seeds *in vitro,* Sardinha *et al.* (1993) washed the seeds under running water for 10 min and then treated with domestic

bleach at varying concentration for 10-30 min. Seeds were germinated in solidified agar (0.3%) or MS at varying strengths with BA (8-10 mg l^{-1}). At 30-50 per cent of bleach 66 per cent of the cultures germinated were free from contamination. On agar and MS full-strength the germination obtained was maximum (83%).

Das *et al.* (1996) germinated both mature and immature nuts by dipping in 70 per cent alcohol for 1 min and flaming, followed by surface sterilizing with 0.1 per cent mercuric chloride for 1 hour. After rinsing the nuts in sterile distilled water, the nuts were again flamed after dipping in 70 per cent alcohol and cut open aseptically to dissect out the entire seed. The seeds were germinated on basal MS medium supplemented with 1 per cent AC and 0.8 per cent agar.

At the National Research Centre for Cashew (NRCC), Puttur, India, freshly harvested mature seeds were first dipped in 70 per cent alcohol and flamed followed by a thorough washing in sterile distilled water with a pinch of detergent and fungicide. The seeds were first sterilized in 70 per cent alcohol (30-45 sec) followed by 0.l per cent mercuric chloride (5-20 min) by constant agitation. After rinsing the seeds thrice in sterile water they were inoculated into screw cap bottles containing media or wet absorbent cotton. Germination is done under dark and is observed after a fortnight. Alternatively, after dipping the seeds in 70 per cent alcohol and flaming them, the testa was removed by shelling the nut in a pedal-operated shelling machine. The whole seed with testa or without testa was sterilized by dip in 70 per cent alcohol followed by 0.1 per cent mercuric chloride (10 min) and inoculated onto B5/MS medium supplemented with 0.1 per cent AC. To germinate immature nuts (4 to 5 weeks old), the green seed was wiped in cotton wetted with alcohol and washed thoroughly in sterile distilled water by adding a pinch of detergent. Sterilization of seed was done first in 70 per cent alcohol (30-45 sec) followed by agitation in 2.5 per cent chlorine solution containing one drop of Tween-20 for 30 min. After its thorough rinsing in sterile

distilled water, the green nut was held with long forceps in a big petri plate, and a transverse cut was given at the centre of it to separate proximal and distal portion of the seed with shell. The distal portion was discarded and from the proximal portion the seed portion with growing point (embryo with a portion of cotyledon) was scooped out and inoculated on to various solid media (MS, WPM, B5) containing 0.1 per cent AC and with or without NAA (2 mg l^{-1}). The incubation was done in 2000-3000 lux light with 16/8-hrs photoperiod. The medium containing WPM salts with NAA was the best with 98.4 per cent germination (Thimmappaiah *et al*, 2001).

Ananthakrishnan *et al*. (2002) obtained *in vitro* germination of mature nuts by culturing the seeds in MS medium supplemented optimally with 22.2 µM BAP and 3 per cent sucrose. After thorough washing of mature nuts in distilled water, surface sterilization was done in 70 per cent ethanol for 15 min and rinsed three times in sterile distilled water. The seeds were decoated and the embryonal axis was gently removed from the cotyledons. Cotyledons were excised and cut in to two transverse halves, i.e. proximal and distal and inoculated in MS basal media supplemented with BAP.

At the KAU, Vellanikkara, freshly harvested, mature seeds were first surface sterilized and then the seed coat and the testa over the embryo were removed. The embryo with the part of the cotyledon was inoculated into solid MS medium supplemented with charcoal, 3 per cent sucrose, kinetin, NAA, brassinolide and cultured at 28±1°C and 12 hours photoperiod provided by cool white fluorescent lamps (2000 lux) (Keshavachandran, 2004).

2.2.1.5 Basal media

Murashige and Skoog medium (1962) can be used for micropropagation of *Anacardium occidentale* (Keshavachandran and Khader,

1990; Hedge *et al*., 1991; D'Silva and D'Souza, 1993; Sardinha *et al*., 1993; Das *et al*., 1996; Ananthakrishnan *et al*., 2002). Keshavachandran and Khader (1990) reported SH medium for micropropagation of cashew. Hedge *et al*. (1991) also used LS (Lin and Staba, 1961) media for micropropagation of cashew. Mantell *et al* (1998) and Boggetti *et al*. (1999) found MS medium with half strength major nutrients ideal for culture establishment of explants derived from glass house raised plants.

2.2.1.6 Carbon source

Many of the workers used sucrose as the carbon source (Ball, 1950; D'Silva and D'Souza, 1992a; Boggetti *et al*., 1999; Thimmappaiah *et al*., 2002; Ananthakrishnan *et al*., 2002).

2.2.1.7 Organic supplements

Thimmappaiah *et al*. 2002 found that organic supplements like L-glutamine showed a significant effect on shoot growth of explants derived from young trees.

2.2.1.8 Shoot bud culture

In shoot culture nodal, shoot-tip and cotyledonary node explants excised from young cashew seedlings have been cultured. Multiple shoots (axillary shoot-bud proliferation) have been observed. Among these cotyledonary node explants have shown the maximum number of multiple shoots. However, the explants derived from mature trees showed poor response.

2.2.1.8.1 *Cotyledonary* node *culture*

Lakshmi-Sita (1989) cultured cotyledonary nodal explants and

obtained 10-13 shoot initials all round the node. They grew to about half a cm in length and stopped with no further growth. Even after transferring the cultures to a medium with BA (0.5 mg l^{-1}) and 3 per cent sucrose, no further growth was observed. Similarly, an average of 3-5 shoot-buds/ cotyledonary nodes was observed on SH medium supplemented with 5-10 mg l^{-1} BA and 0.5 mg l^{-1} NAA (Keshavachandran and Khader, 1990). Multiple shoot-buds were induced in cotyledonary nodes cultured along with cotyledonary nodes on MS medium supplemented with 4 per cent sucrose, 5.3 g l^{-1} maltose and 5 mg l^{-1} BA. The shoot-buds elongated on MS medium supplemented with CW (10%), 5.3 g l^{-1} maltose and 5 per cent sucrose (D'Silva and D'Souza, 1992a, b).

Das *et al.* (1996) observed that cotyledonary nodes produced more buds (12 buds) than the other explants on MS medium containing 1 mg l^{-1} BA, 0.5 mg l^{-1} Kin and 2.0 mg l^{-1} zeatin.

Cotyledonary nodes with intact cotyledons obtained from *in vitro* germinated seedlings (mature seed) of H4-7 and VRI-2 showed multiple shoot induction on MS medium supplemented with 2.25 mg l^{-1} BA and 0.2 mg l^{-1} IBA. After 5-6 subcultures at monthly intervals, the shoot-bud proliferation increased and as many as 40-60 shoots could be obtained in a span of 3-4 months. Cotyledonary nodes without their cotyledons also showed shoot-bud proliferation (4-6 shoots/explant) on basal medium without any supplementation of hormone and with 0.5 mgl^{-1} zeatin on MS medium (Thimmappaiah, 1997).

2.2.1.8.2 Node and shoot-tip culture

Nodal segments excised from the 6 to I5-month-old seedlings showed bud bursting on Lepoivre medium containing MS minor nutrients, glucose (3%), ascorbic acid (0.1 %), 2iP (2 mg l^{-1}) and GA_3 (0.5 mg l^{-1}). On the medium containing Kin (2 mg l^{-1}) also, the axillary shoot development was similar. Although there was opening of bud in presence of BAP medium, there

was limited development of the axils. Proliferation was stimulated by the two phase technique and enhanced with the addition of BAP (0.5 mg l^{-1}) in the liquid phase for at least three days. Continuous presence of BAP in liquid phase proved toxic as the nodes became necrotic. The average number of axillary shoots per node was 2-3. Elongation of shoots was obtained on liquid medium under agitation with vitrification of shoots (59%).

Nodal segments on modified MS and LS medium supplemented with BA (2.0 mg l^{-1}) and GA_3 (1.0 mg l^{-1}) showed only a single axillary shoot formation in two months. Initially, the cultures were kept in the dark for one month. The shoot length attained was 1.5 - 1.75 cm. No rooting could be obtained from such shoots. The shoot-tips from seedlings and air-layered plants were induced to grow only when cultured on MS and LS medium supplemented with 5-10 mg l^{-1} IBA and 1 per cent AC, under continuous darkness for 4 weeks. Slight callus formation at the base was observed on proliferating medium containing cytokinins alone. But the proliferation media containing cytokinins with low levels of auxin showed slight proliferation after two to three months (Hedge, 1988).

At Central Plantation Crops Research Institute, Kasargod, India, apical meristem and axillary buds of nodal region excised from mature trees were cultured on MS and elongation of shoots obtained and also observed formation of a compact, white or creamy callus at the cut-end. Further induction and growth of callus required dark incubation in the medium containing 2 mg l^{-1} 2,4-D (Nambiar and Iyer, 1988).

Shoot-tips and nodal cuttings excised from the young seedlings showed no good response when they were cultured on SH medium supplemented with 1 mg l^{-1} BAP, 1 mg l^{-1} Kin, 0.5 mg l^{-1} GA_3 or with 0.5 mg l^{-1} BAP + 0.2 mg l^{-1} Kin + 0.1 mg l^{-1}calcium pantothenate + 0.1 mg l^{-1} biotin (Lakshmi-Sita, 1989).

Lievens *et al* (1989) reported that nodal segments excised from the 6 to 15 month old seedlings showed bud bursting on Lepoivre medium containing MS minor nutrients, glucose (3%), ascorbic acid (0.1%), 2iP (2 mg l^{-1}) and GA_3 (0.5 mg l^{-1}). On the medium containing Kin (2 mg l^{-1}) also, the axillary shoot development was similar. Although there was opening of bud in presence of BAP medium, there was limited development of the axils. Proliferation was stimulated, by the two phase technique and enhanced with the addition of BAP (0.5 mg l^{-1}) in the liquid phase for at least three days. Continuous presence of BAP in liquid phase proved toxic as the nodes became necrotic. The average number of axillary shoots per node was 2-3. Elongation of shoots was obtained on liquid medium under agitation with vitrification of shoots (59%).

Keshavachandran and Khader (1990) overcame polyphenol oxidation in the explants by: (1) washing the explants in running water for two hours, (2) agitation in PVP solution (0.5% w/v) prepared in 2 per cent sucrose for 45 min at 100 rpm followed by three immediate rinsing in ascorbic acid and citric acid (50: 75 mg l^{-1}) mixture, (3) culturing initially in the dark for 3 weeks, and (4) supplementing the media with 0.5 per cent activated charcoal. SH medium supplemented with 6.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA and Linsmaier and Skoog's (1965), (LS) medium were found ideal for nodal cultures and induced 3-4 shoots/explant with an average of 2.1 buds. Addition of adenine sulphate (80 mg l^{-1}) or CW (15%) was beneficial for increasing the rate of multiplication. Activated charcoal at 1 g l^{-1} was found to be essential during proliferation and rooting. Varietal differences were observed in multiplication rate, maximum being in cultivar BLA-39-4. Regeneration from the shoot-tips was not as good as nodes. Keshavachandran and Khader (1990) reported shoot-bud proliferation (3-4 buds) from shoot-tips on MS and LS media.

Shoots from microcuttings of cashew were cultured on half-strength MS and SH media supplemented with zeatin riboside (5 mg l^{-1}), BA, Kin, 2iP, GA_3, TIBA and NAA alone and in combination. Only MS medium

supplemented with 5 mg l^{-1} zeatin riboside supported the axillary bud growth. The shoots cultured on SH medium supplemented with zeatin appeared yellowish with lamina rolled, whereas MS with other hormones alone did not support bud growth. Shoots in the presence of zeatin riboside first showed a 'time-lag' in growth followed by elongation and node formation without successive opening of the new axillary buds indicating apical dominance. GA_3 and TIBA (0.5 mgl^{-1} each) were tried for overcoming apical growth inhibition. Gibberellic acid (GA_3) in combination with zeatin riboside favoured shoot elongation and bud growth (Leva and Falcone, 1990).

Nodal cultures were established on half-strength MS medium containing BA with IAA or NAA on both agar-solidified medium and liquid medium supported with filter paper bridges. In liquid medium, the phenolic exudation was less, and the shoots remained green with some callus formation. Budding in 85 to 100 per cent cultures was observed after two weeks in culture (Bessa, 1990).

Farrant *et al.* (1990) observed shooting of apical nodes cultured on woody plant medium (Llyod and McCown, 1980) supplemented with BA. Rooting was less uniform and observed clonal variation was observed for rooting response.

Multiple shoots from leaf axils of seedlings were induced on modified MS containing ammonium nitrate (2000 mg l^{-1}), calcium chloride (650 mg l^{-1}), potassium dihydrogen orthophosphate (200 mg l^{-1}), 4 per cent sucrose, CW (10%), 10 mg l^{-1} BA and 4.8 mgl^{-1} Kin. The buds formed showed no elongation (D'Silva, 1991).

Nair and Mohanakumaran (1993) cultured nodal segments on SH medium with either NAA (2 mg l^{-1}) + Kin (2 mg l^{-1}) + AC (0.1 %) and incubated in the dark for 3 weeks at 26°C or with BA (2 mg l^{-1}) + NAA (2 mg l^{-1}) + sucrose

(2%) medium. Shoots got elongated when the cultures were incubated in light (3000 lux) in a 16 h photoperiod. The shoots attained a length of 3.02-4.0 cm with 2-5 leaves. The elongated laterals were multiplied on either SH + BA (0.5 mg l^{-1}), or SH + BA (1.0) + Kin (1.0 mg l^{-1}) + IBA (0.2 mgl^{-1}) + GA_3 (1.0 mgl^{-1}) or SH + BA (2 mg l^{-1}) + IAA (1.0 mg l^{-1}) + AC (1%) + myoinositol (l00 mg l^{-1}). However, they did not specify the multiplication achieved. They observed reduction in the phenolic exudation by shaking the explants in PVP (7.0 g l^{-1}) solution for 30-40 minutes and by including 10 g l^{-1} AC in the initiation medium and incubating the cultures in darkness for a minimum period of three weeks.

Age and plant growth conditions influenced the rate of multiplication in nodal cultures (Sardinha *et al.,* 1993). Maximum bud break (94%) was noticed on hormone-free MS medium. Better flushing and multiple shoot induction was observed on the medium containing BA (1.0 mg l^{-1}) and IAA (0.5 mg l^{-1}). BA and NAA had shown some inhibitory effect. Multiple shoots were observed from the callus produced at the base of 9 to 12 month-old *in vitro* cultures. Reduction of phosphates or nitrates in standard MS medium had no impact on the results.

Nodal segments of seedlings showed axillary buds and multiple shoot development on semi-solid MS medium modified in respect of nitrogen concentration and supplemented with 44 µmol L^{-1} GA_3. Functional leaves developed within 3-4 weeks of incubation at 26°C, 3000 lux illumination, 14:10-hrs day and night cycle and 80 per cent RH (Lad *et aI.,* 1994).

In vitro multiplication of cashew was achieved when callus induced at the base of the microcuttings was sub-cultured at monthly intervals on MS mineral medium containing Morel's vitamins. A medium with BA (1 mg l^{-1}) and IAA (0.6 mg l^{-1}) and another medium free of hormones were used. After 9-15 months, the calli started to produce multiple flushes at a high frequency. The white callus could be multiplied easily on hormone-free medium. The shoots were

rooted spontaneously or by an auxin treatment (Bessa and Sardinha, 1994).

Das *et al.* (1996) observed shoot multiplication in nodal (4 buds) and shoot-tip explants excised from the seedlings raised from immature embryos were cultured on MS medium supplemented with BAP (1 mg l^{-1}) + Kin (0.5 mg l^{-1}) + zeatin (2 mg l^{-1}).

Nodal cultures made from one-month-old seedlings and *in vitro* germinated seedlings showed satisfactory establishment on ¾th salt MS medium with 75 per cent of the explants forming single shoots. However, the shoot-tips from nursery seedlings showed poor response. Nodal cultures grown on MS medium with various levels of BA, Kin, 2iP, adenine sulphate showed only single shoot formation. Even incorporating BA, Kin, 2iP and zeatin in combination of two at two levels (0.5 and 1.0 mg l^{-1}) produced very low multiple shoot induction (6-18%) (Thimmappaiah, 1997). However, when shoot-tips and nodal cuttings excised from *in vitro* source were cultured on the above basal MS medium supplemented with thidiazuron (TDZ) alone (0.05 - 2 mg l^{-1}) or in combination of TDZ at 0.1 mg l^{-1} with BA (0.5, 1.0 mg l^{-1}), IBA and NAA (0.05-0.5 mg l^{-1} each), the multiple shoot production (1-13 shoot buds/explant) was induced in majority of the explants. When TDZ was used alone, the multiple shoot-buds (1-5 buds/explant) were obtained with an average of 1.7 shoot-buds/explant, and when TDZ was combined with other plant growth regulators, the multiple shoot-bud formation increased to an average of 3 buds/explant. The best combination for multiple shoot induction was TDZ (0.1 mg l^{-1}) with either 0.1 mg l^{-1} of IBA or NAA with an average of over 4 shoot-buds/explant (Thimmappaiah and Shirly, 1996, 1999).

The shoot-buds induced could be elongated on half MS or on Raj Bhansali (1990) solid medium supplemented with 400 mg l^{-1} glutamine and activated charcoal (0.2%). Even liquid MS medium supplemented with 2.5 mg l^{-1} Kin shaken at 100 rpm and in light was useful for elongation of shoot-buds

(Thimmappaiah, 1997; Thimmappaiah and Shirly, 1996, 1999).

Nodal cuttings from forced shoots (pruned frames) of cashew grafts (one-year-old) and field-grown trees (10-year-old) could be established on semi-solid half-strength MS salt medium supplemented with glutamine (400 mg l^{-1}) and AC (2 g l^{-1}). Budding of explants was affected by age of the plant. Establishment of cultures was also affected by excessive amount of contamination and tissue browning. These shoots on subculturing in MS or WPM supplemented with 0.1 and 2.0 mg $l^{-1}$of TDZ showed multiple shoot induction (2-6 buds/explant). However, they failed to show elongation. Slow growth and drying (necrosis) of shoots were other problems encountered during the culture of mature tree explants (Thimmappaiah, 1997).

Experiments were conducted to investigate cultural factors affecting bud sprouting, shoot elongation and node development from shoot nodes of elite selections of one month-old and 1-5-year-old greenhouse-raised cashew stock plants originating from Brazil, Guinea-Bissau and Tanzania. MS medium containing half-strength macro elements was the most effective medium for bud sprouting and shoot elongation. The presence of gibberellic acid in association with zeatin or 2iP promoted flush growth, while in association with 2iP or Kin it favoured shoot elongation (Mantell *et al.*, 1998).

Using glasshouse-raised plants (1 month, 1 year and 5 years old), factors affecting shoot development from shoot nodes of 1 Tanzanian and 2 Brazilian elite selections of cashew were assessed by Boggetti *et al.* (1999). Sprouting of buds decreased strongly with increasing age of mother plants. MS salts containing half-strength macroelements were the most suitable for bud sprouting and shoot elongation. Gibberellins supported bud sprouting and shoot elongation but blocked rooting. The shoots developed in the presence of cytokinins were short and produced axillary branches.

A study was conducted by Mneney and Mantell (2002) to develop *in vitro* culture techniques relevant to the clonal multiplication of cashew using the Tanzanian elite clone AC4. The results showed that MS medium with full strength macroelements was best for shoot proliferation and elongation and also the ability of axillary buds of juvenile explants (1-6 months old) to sprout and elongate increased compared to those from aged explants. Out of the 6 cytokinins compared, BA and zeatin were found to be optimal for axillary shoot proliferation and shoot elongation respectively. When cultured on woody plant medium with 0.5 mM IBA, 50-60 per cent of microshoots rooted *in vitro,* and about 60-65 per cent survived weaning and produced healthy vigorously growing plants.

Keshavachandran (1996), Keshavachandran and Shelja (1999), Keshavachandran (2004) and Keshavachandran and Riji (2005) reported that shoot tips, nodal segments and cotyledonary nodes taken from *in vitro* raised seedlings were used to establish *in vitro* cultures. The explants were isolated 7-10 days after germination of mature seeds under *in vitro* conditions and cultured in appropriate media. The explants were best established after culturing in the dark for 7 days in MS medium supplemented with kinetin, NAA and brassinolide. They initiated growth when cultured under light after 7 days in the dark. The cultures after 3 weeks under light were transferred to MS medium supplemented with coconut water, maltose, sucrose and brassinolide for another three weeks. The shoot buds taken from grafted plants were initially cultured in MS medium supplemented with kinetin, NAA, brassinolide, activated charcoal and myo-inositol and kept in the dark for 7 days. After 3 weeks under light, the cultures were subcultured into MS medium supplemented with BA, branssinolide, activated charcoal and myoinositol and cultured for 3-4 weeks.

From the reports, it is observed that various basal media (MS, SH. LS, WPM, Lepoire) with hormonal supplements helped in establishment of shoot cultures. Among the various basal media, SH and MS media (at reduced levels of

salt) supported better for the culture establishment of explants from seedling origin. Among the hormonal supplements, BA, zeatin and thidiazuron alone or in combination with others were conducive for inducing axillary shoot-bud proliferation. However, attempts on regeneration from the field-grown mature trees are few and far from satisfactory with no complete formation of plant. Hence, tissue culture in cashew is considered as intractable and recalcitrant.

2.2.1.8.3 Rooting of microshoots

Rooting was obtained in 30 per cent shoots on IBA (2 mg l^{-1}) containing medium following ten days of dark incubation (Lievens *et al.*, 1989). Keshavachandran and Khader (1990) reported rooting of microshoots was achieved on half-strength LS and SH media supplemented with NAA (2.5-5.0 mg l^{-1}) and 12 hrs photoperiod. The rooted plantlets survived only up to 29 days in pots. NAA at 5 mg l^{-1} resulted in 25 per cent rooting with the formation of basal callus (Leva and Falcone, 1990). The microshoots were rooted in *in vitro* on MS medium with 4 per cent sucrose, 0.5 mg l^{-1} IAA and 1 mg l^{-1} IBA, and the plantlets were potted and established in the field (D'Silva and D'Souza, 1992a, b).

Nair and Mohanakumaran (1993) reported that *in vitro* rooting of single shoots was observed on half-strength MS medium containing NAA (2.0-5.0 mg l^{-1}), or IBA (2.0 mg l^{-1}) or NAA (2.0 mg l^{-1}) + IBA (2.0 mg l^{-1}). The best rooting (50%) was seen on the medium containing NAA (2.0 mg l^{-1}) + IBA (2.0 mg l^{-1}) + sucrose (3%) + agar (0.6%) + AC (1 g l^{-1}). For the first fifteen days, the shoots were placed in auxin medium, and subsequently the rooted ones were transferred to half-strength MS without phytohormones. The number of roots formed varied from 1-5 per shoot and the root length from 6.0-8.0 cm in 14 days after initiation. Attempts to induce *ex vitro* rooting by a slow dip in IBA (100 mg l^{-1} for 24 hours), or a quick dip in IBA (2000 mg l^{-1} for 10 seconds) were not successful.

Tap root formation was reported from the bottom end of nodal segments on modified semi-solid Lloyd's medium supplemented with BA (2.5 mg l^{-1}), NAA (2.8 mg l^{-1}), GA_3 (3.7 mg l^{-1}) and AC (0.1 %) for one week of continuous dark incubation followed by two weeks of normal incubation in light (Lad *et aI.,* 1994).

Das *et al.* (1996) observed rooting of microshoots increased to 80-90 per cent when the microshoots were submerged in a suspension of *Agrobacterium rhizogenes* as observed with chronic treatment of IBA against 20-40 per cent rooting.

Rooting was induced spontaneously and by auxin treatment with IAA or IBA. However, rooting results were not consistent. *Ex vitro* rooting by IBA dip was also observed though the frequency was low (Sardinha *et al.,* 1993).

NAA at l mg l^{-1} or at lower levels was favourable for rooting (Nambiar and Iyer, 1988). The elongated shoots (+2 cm) could be rooted both by *in vitro* and *ex vitro* methods. *In vitro* rooting tried with half MS solid medium supplemented with 3 per cent sucrose and auxins NAA, IBA, IAA at 2.5, 5.0 mg l^{-1} each and in combination of two at 2.5 mg 1^{-1} each showed maximum rooting (50%) in combination containing 2.5 mg 1^{-1} each of NAA and IBA and in NAA alone at 5.0 mg1^{-1}. The total duration for rooting was 10 to 40 days with 1-6 roots per plant. No dark treatment was required for rooting. There was variable response to solid and liquid medium. Liquid medium was found superior to solid medium, and hormone medium was better than hormone-free medium for rooting. *Ex vitro* rooting was also observed to a lesser degree (12.5%) in microshoots treated/dipped in 250 ppm of NAA or lAA alone for 48 hours in the dark and potted in soilrite and sand mixture pots (Thimmappaiah and Shirly, 1996).

Microshoots derived from 1-2 month old, 1-5 year old trees rooted in *vitro* at a frequency of 42 per cent when cultured for 5 days on WPM

supplemented with 100 µmol IBA. Almost 100 per cent of rooted shoots survived weaning and produced healthy, vigorous plants (Mantell *et al.*, 1998). Microshoots derived from 1 month old, 1-5 year old rooted in *vitro* at a frequency of 42 per cent when cultured for 5 days with 100 µmol IBA. Over 40 per cent of rooted microshoots survived weaning (Boggetti *et al.*, 1999). Microshoots cultured on woody plant medium with 0.5 mM IBA, 50-60 per cent of microshoots rooted *in vitro*, and about 60-65 per cent survived weaning and produced healthy vigorously growing plants (Mneney and Mantell, 2002).

In vitro rooting was found feasible with pulse treatment with IBA for 24 hours followed by transfer to ½ MS liquid medium supplemented with low levels of IBA and reduced sucrose. Rooting was also obtained with ¼ MS medium supplemented with glutamic acid, IBA and reduced sucrose shoots grafted plants were pulse treated with high concentration of IBA followed by transfer to ¼ MS liquid medium supplemented with IBA, glutamic acid, reduced sucrose and myo-inositol (Keshavachandran and Riji, 2005).

2.2.1.8.4 Hardening and planting out

The rooted plantlets after one week's exposure to light were transferred to pots containing sterile sand (Nair and Mohanakumaran 1993). On a pot mixture containing lime-free-based compost and perlite, 83 per cent plantlets survived the hardening process (Sardinha *et al.*, 1993).

Rooted plantlets were hardened in the laboratory on liquid MS medium with gradual reduction of sucrose and also in pots containing sand and soilrite mixture 2:1 covered with polybags. In laboratory, they had a survival of 80 per cent. Later the hardened plants from the laboratory were potted in normal pot-mixture and housed in the greenhouse for further hardening under diffuse light. From explant to planting of tissue culture plants, a total duration of 38-52

weeks was required. Regenerated plants were planted in the field with 100 per cent establishment (Thimmappaiah and Shirly, 1996, 1999).

Hardening was initiated under *in vitro* conditions itself by cutting the rooted plantlets in liquid ½ MS containing low levels of sucrose for 10 days and then in medium without sucrose for another 10 days. The rooted plantlets were taken out of the containers, washed with distilled water to remove the adhering media, dipped in fungicide for 5 min and transplanted into small mud pots filled with sterilized sand which were immediately transferred into the mist chamber. After around 45-60 days in the mist chamber, the plantlets were transferred into medium sized mud pots filled with normal potting mixture and kept in the net-house until planting in the field (Keshavachandran and Riji, 2005).

2.3 BACTERIAL ENDOPHYTES IN *IN VITRO* CULTURES

Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Holliday, 1989; Schulz & Boyle, 2006), and of the nearly 3,00,000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel *et al.*, 2004). James and Olivares (1997) modified the definition and stated that all bacteria that colonize the interior of plants, including active and latent pathogens, can be considered to be endophytes.

Many of them are able to transcend the endodermis barrier, crossing from the root cortex to the vascular system, subsequently thriving as endophytes in stem, leaves, tubers and other organs (Bell *et al.*, 1995; Compant *et al.*, 2005a). The internal tissue of plants provide uniform and safe environment when compared to rhizosphere and phylloplane where the introduced bacterial population must compete for nutrients and also endure temperature changes and exposure to UV rays. Endophytic bacteria inhibit plant pathogens by production

of antimicrobial compounds like antibiotics and siderophores (Leyns *et al*., 1990). Some of them are capable of inducing systemic resistance in plants (Kloepper *et al*., 1992). Endophytic bacteria that colonize the internal plant parts are reported to have several beneficial effects on the plants such as resistance against pathogens and favour plant growth (Kloeppar *et al*., 2004). Inside the plant tissue endophytic bacteria remain localized in specific plant tissues like the root, cortex or colonize systematically by transport through conducting elements (Kloepper *et al*., 1992a).

2.3.1 Source of bacterial endophytes

Bacterial endophytes are consistently reported to be present in the root, stem, leaf, fruit and tuber tissues of a wide range of agricultural, horticultural, and forest species. Bacteria found within roots, shoots, leaves, seeds and ovules are usually similar to those found in adjacent root zone soils (Hollis, 1951; Holt, 1994; Lamb *et al*., 1996; Mclnroy and Kloepper, 1995; Mundt and Hinkle, 1976) supporting the view that soil is a major source from which endophytic bacterial populations originate.

2.3.2 Host entry of bacterial endophytes

The highest bacterial densities are usually observed in the roots and decrease progressively from the stem to the leaves (Quadt-Hallman and Kloepper, 1996; Lamb *et al*., 1996). The root is the primary site where endophytes gain entry into plants. With the exception of seed transmitted bacteria which are present in the plant, potential endophytes must first colonize the root surface prior to entering the plant. In plants that propagate vegetatively, such as potatoes, parent material (eg. tubers) can be a source of endophytic bacteria that subsequently colonize the developing roots and shoots via vascular tissues. Potential bacterial endophytes may be moved from the soil to the host plant by a number of mechanisms, including wind action, attachment to soil particles, in

water on agricultural equipment by vectors including humans, birds, mites (Bashan, 1986), insects (Armstrong *et al.*, 1987; Schalk *et al.*, 1987) and bacterial feeding nematodes (Kimpinski and Stur'z, 1996).

Host entry points identified during bacterial colonization include stomata hydathodes (Cook *et al.*, 1952) nectarthodes, lenticels (Fox *et al.*, 1971; Scott *et al.*, 1996) germinating radicles, tissue wounds associated with the emergence of secondary roots, via broken trichomes (Lamb *et al.*, 1996; Jacobs *et al.*, 1985) wounds sustained during root growth through soil (Daft and Leben, 1972) foliar damage from wind blown soil particles, rain or hail through undifferentiated meristamatic root tissues (Hollis, 1951).

2.3.3 Location of endophytic bacteria

Once inside the plant tissue, endophytic bacteria remain localized in a specific plant tissue, such as the root cortex or colonize the plant systematically by transport or active migration through the conducting elements or the apoplast (Hurek *et al.*, 1991; Mahaffee and Kloepper, 1997; Quadt-Haltmann *et al.*, 1997; Patriquin and Doberrinner, 1978). Internal colonization of plant tissues by bacteria is considered to be primarily intercellular, with most reports stressing the importance of xylem vessels as reservoirs of internal populations of bacteria (Gardner *et al.*, 1982; Jacobs *et al.*, 1985; Frommel *et al.*, 1991; Kloepper *et al.*, 1992; Bell *et al.*, 1995; Dong *et al.*, 1994) reported the presence of endophytic bacteria from the intercellular spaces of sugarcane stem parenchyma. Although less fully documented intracellular endophytic bacteria have also been found within the cytoplasm and vacuoles of cell walls including epidermal cells (Quadt-Hallmann and Kloepper, 1996), root hairs (Vance, 1983) and parenchyma cells (Jacobs *et al.*, 1985).

2.3.4 Isolation and characterization of bacterial endophytes

The endophytic niche offers protection from the environment for those bacteria that can colonize and establish in planta. These bacteria generally colonize the intercellular spaces, and they have been isolated from all plant compartments including seeds (Posada & Vega, 2005). Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species, such as oak and pear, to herbaceous crop plants such as sugar beet and maize. Classical studies on the diversity of bacterial endophytes have focused on characterization of isolates obtained from internal tissues following disinfection of plant surfaces with sodium hypochlorite or similar agents (Miche & Balandreau, 2001). A review by Lodewyckx *et al*. (2002) highlights the methods used to isolate and characterize endophytic bacteria from different plant species. A comprehensive list of bacterial endophytes isolated from a broad range of plants is provided by Rosenblueth and Martinez-Romero (2006) and Berg and Hallmann (2006), which updates the groundwork laid by Hallmann *et al*. (1997) and Lodewyckx *et al*. (2002).

Molecular approaches for the isolation and characterization of bacterial endophytes and plant-associated bacteria and communities have been reviewed recently by Franks *et al*. (2006). Microbial communities inhabiting stems, roots and tubers of various varieties of plants were analysed by 16S rRNA gene-based techniques such 16S rRNA gene cloning and sequencing. Five taxa exhibiting the most promising levels of colonization and an ability to persist were identified as *Cellulomonas*, *Clavibacter*, *Curtobacterium*, *Pseudomonas* and *Microbacterium* by 16S rRNA gene sequence, fatty acid and carbon source utilization analyses (Elvira-Recuenco and Vuurde, 2000; Zinniel *et al*., 2002). 16S rDNA was amplified by two universal primer: 16S $_{43\text{-}63}$ and 16S $_{1404\text{-}1387}$ (Radeva and Selenska-Pobell, 2005). High densities of endophytic bacteria were found in plant material from poplar, larch and spruce that had been micropropagated for at least 5 years. The majority of these bacteria were assigned to the genus

Paenibacillus based on the sequencing of the 16S rRNA genes (Ulrich *et al*., 2008). Fourteen distinct bacterial clones were isolated from surface sterilized shoot tips of Papaya (*Carica papaya*) plated on MS during 2-4 week period following *in vitro* culturing. These isolates were ascribed to six Gram-negative genera namely Pantoea (*P. ananatis*) Enterobacter (*E. cloacae*), Brevundimonas (*B. auriaca*), Sphingomonas, Methylobcterium (*M. esteraromaticum*) and Bacillus (*B. benzoevoeas*) based on 16S r DNA sequence analysis (*Thomas et al*., 2007). Identification of endophytic bacteria *Enterobacter, Klebsiella, Ochrobactrum*, *Pantoea*, *Staphylococcus* and *Bacillus* spp. isolated from shoot tip cultures of banana cv. Grand Naine based on partial 16S rRNA gene sequence homology analysis (Thomas *et al*., 2008a; Thomas *et al*., 2008b).

2.4 COVERT BACTERIA IN *IN VITRO* CULTURES

Latent or covert bacterial contamination is a serious problem in plant tissue cultures (Leifert and Woodward, 1998); Leifert and Cassells, 2001). The bacteria gain entry in cultures as epiphytes, endophytes or incidental contaminants and may survive in them in a totally unsuspecting manner warranting culture indexing for their detection (Viss *et al*., 1991; Thomas, 2004a). It is suggested that the term 'covert bacteria' as described by Holland and Polacco (1994) or Horsch and King (1983) is more appropriate than the frequently used term 'latent bacteria' to describe such bacteria which are not normally visible on tissue culture medium. Presence of covert bacteria in the cultures is highly undesirable due to obvious adverse effects on growth (Leifert and Waites, 1992; Thomas, 2004), lack of reproducibility of tissue-culture protocols (Thomas, 2004a), possible hormone-mediated growth effects (Holland and Polacco, 1994), ramifications in cell cultures (Horsch and King, 1983), possibility of carrying pathogens (Cooke *et al*., 1992), potential risk to *in vitro* gene banks (Houwe and Swennen, 2000) and safe exchange of germplasm (Salih *et al*.,2001). All these reduce the reliability of plant cell/tissue-culture systems (Leifert and Cassells, 2001; Thomas, 2004a). Availability of a reliable screening method is the primary

requirement for tackling the covert contamination problem. Many studies have addressed the isolation and identification of common bacteria in tissue cultures (Houwe and Swennen, 2000; Tanprasert and Reed, 1997).

Testing the cultures using bacteriological indexing medium (BIM) is the simplest and best method as it allows non-specific detection of a wide range of bacteria even when present in low numbers (Leifert and Woodward, 1998; Cassells, 2000). Molecular and serological methods allow detection of specific organisms but they are expensive, expertise-demanding and of limited use for general indexing (Leifert and Woodward, 1998; Stead *et al*., 2000).

Conventional indexing methods include incorporation of bacterial growth enhancing constituents in the tissue culture medium (Boxus and Terzi, 1987), placing the tissue in enriched liquid medium and assessing its turbidity (Tanprasert and Reed, 1997; Niedz and Bausher, 2002) or streaking the base of plantlets on BIM (Viss *et al*., 1991; Houwe and Swennen, 2000). Placing pieces of tissue on BIM (Leifert and Woodward, 1998) or testing the tissue homogenate on BIM (Kamoun *et al*., 1998) has been suggested to detect any endophytic bacteria that survived initial decontamination treatment. Covert bacteria-harboring long-term micropropagated cultures of grape that was sanitized of associated microbes as assessed through the indexing of tissue and medium for several passages (Thomas and Prakash, 2004) showed re-emergence of bacteria in a section of cultures (3/40) during the subsequent screening. The presence of latent bacteria is a serious problem in plant tissue cultures. While endophytes are generally beneficial to plants *in situ*, they may affect culture growth under the modified conditions *in vitro*. Lata *et al*. (2003) identified and characterized endophytic bacteria associated with the medicinal plant *Echinacea* in tissue culture. Panicker *et al* (2007) reported that the presence of covert endophytic bacteria in apparently clean cultures of Chrysanthemum.Thomas *et al*. (2007b) reported widespread prevalence of fastidious or viable but non-culturable

endophytic bacteria in field shoots and in unsuspicious shoot-tip cultures of papaya (*Carica papaya* L.) against the norm of asepsis *in vitro*.

2.5 VESICULAR ARBUSCULAR MYCORRHIZAL ASSOCIATION IN CASHEW

Cashew is generally grown in poor and marginal soils but responds well to applied nutrients. Vesicular arbuscular mycorrhiza (VAM), the symbiotic fungi associated with the roots of most annuals as well as perennial plants help them in the uptake of phosphorus and micronutrients at the expense of carbohydrates synthesized by higher plants (Krishna and Bagyaraj, 1983; Bopaiah *et al.*, 1989). The main advantage of mycorrhiza lies, through extension of the penetration zone of the root fungus system in soil, facilitating an increased efficiency of water and nutrient uptake by the host plant. The interconnected network of external hyphae acts as an additional absorbing surface in the soil beyond the zone of root exploration.

Micropropagated plantlets inoculated with active culture of VAM enhanced their establishment and growth (Varma, 1995). This eliminates the transient shock and stunted growth on transfer to field (Lovato *et al.*, 1996). The most important problem associated with micropropagation is the establishment of *in vitro* derived plantlets under conditions which limits the widespread use of the technology. Tissue culture plantlets show certain aberrant physiological features which lead to very high mortality due to desiccation and microbial infection ethics during hardening or during transfer to filed conditions, which limits the widespread use of the technology. (Capellades *et al.*, 1990).

2.5.1 Physiological features of tissue culture plants

The tissue culture plantlets when planted out undergo desiccation and drying due to poor development of cuticle and epicuticular wax on the newly

emerging leaves, as a result of high humidity in the culture vessels (Leshem, 1983). The palisade cells of leaf surface are poorly developed and have pronounced mesophyll air spaces (Donnelly and Vidaver, 1984). The plantlets are photomixotropic and have leaves with low chlorophyll content and low photosynthetic rates that impede growth due to poor organization of grana in the chloroplasts of the *in vitro* growing plantlets along with etiolated effect produced by ethylene in the glass vessels (Grout and Aston, 1977; Lee *et al.*, 1985). The micropropagated plantlets show an impaired stomatal mechanism (Capellades *et al.*, 1990) due to which transpiration rates are initially very high, which results in wilting, necrosis of leaves and may include senescence and death of leaves and plantlets (Preece and Sutter, 1991). A percentages of cultures show water soaked, almost translucent leaves and exhibits a decline in growth and multiplication (Debergh *et al.*, 1981). Lack of proper root hair development in tissue culture plants in general directly affects the absorption of nutrients and water from the soil (Reuther, 1986). These major physiological effects of tissue culture plantlet affect uptake of water and nutrients from soil and transportation to the shoot system and plantlets become more vulnerable to desiccation and wilting and also to pathogenic infection (Sivaprasad and Sulochana, 2004).

2.5.2 Characteristics of AMF

Arbuscualar mycorrhizal fungi (AMF) are soil fungi which form symbiotic association with plants and colonize roots of most of the plant families (Smith and Read 1997). Arbuscular mycorrhizal associations are formed by a group of Zygomycetous fungi belonging to the order Glomales (Morton and Benny, 1990). It produces vesicles and arbuscules in the cortex of the infected root and penetrates the soil beyond the zone accessible to root. These endophytes are obligate symbionts and spread in association with host plants. VAM infection does not change the external appearance of the root and consists of intercellular distributive hyphae, finely branched intracellular structures, the arbuscules and

spore like swelling and the vesicles usually at the hyphal tip (Bopaiah *et al*., 1989).

They impact a variety of benefit to their hosts, which include increased growth and yield due to enhanced nutrient acquisition (Diedrichs and Moawad, 1993), water relations (Davies *et al*., 1993; Subramanian *et al*., 1995), pH tolerance (Clark and Zeto, 1996) and disease and pest tolerance (Lopez *et al*., 1997). Various laboratory and green house experiments have demonstrated that VAM inoculation can improve growth and nutrition of crop plants (Hayman, 1980; Smith and Read, 1997; Raju *et al*., 1990; Lin and Hao, 1988).

To what extent a plant gets benefited by the mycorrhizal association depends upon its phosphorus requirement. Plant with long and fine roots hairs are less dependent upon VAM than those with short fleshy roots and root hairs as in citrus. In tropical condition, mycorrhizal symbiosis is of major importance for phosphorus nutrition since phosphorus gets easily fixed in soil (Bopaiah *et al*., 1989).

2.5.2.1 Improved nutrient update

The enhanced plant growth due to mycorrhizal colonization is mainly attributed to increased nutrients especially P (Mosse, 1973; Harley and Smith, 1983; Jaizme-vega and Azcon, 1991; Vidal *et al*., 1992; Joseph, 1997; Sivaprasad *et al*., 1999a; Khaliq *et al*., 2001; Estrada-Luna and Davies 2003; Sivaprasad and Sulochana, 2004; Bucher, 2006). This is because fungal hyphae spread in the soil, increase the absorbing surface available for soil nutrient uptake which help in effective exploration of higher volume of soil for nutrients (Rhoder, 1980; Bolam, 1987). The role of AMF in improving the uptake of other nutrients viz, N (Vidal *et al*., 1992; Li *et al*., 2002), Cu (Gildon and Tinker, 1983; Li *et al*., 1991), Zn (Faber *et al*., 1991; Chen *et al*., 2003), Ca, K, Mg (Liu *et al*., 2002) Fe (Caris *et al*., 1998), Cd (Guo *et al*., 1996; Gonzalez-Chevez *et al*., 2002), Ni (Guo

et al., 1996; Jamal *et al.*, 2002) and U (Rufykin *et al.*, 2002) were well documented.

2.5.2.2 Improved water relations

AMF plays an important role in water economy of plants (Safir *et al.*, 1971, 1972; Al-Karaki, 1998) by improving hydraulic conductivity of the root at lower soil water potential (Hardic and Layton, 1981; Dell' Amico *et al.*, 2002; Sanchez-Blanco *et al.*, 2004; Sivaprasad and Sulochana, 2004; Allen, 2007). AM colonization allowed plants to maintain their water content, water potential and leaf transpiration at high levels (Yano-Melo *et al.*, 1999; Sanchez-Blanco *et al.*, 2004). Jeffries *et al.* (2003) showed that AMF are the most important microbial symbioses, which under conditions of P limitation influence plant community development, water relations and above ground productivity.

2.5.2.3 Drought tolerance

AMF association enhances tolerance of plants to toxicity and drought (Atkinson and Davidson, 1972; Guttag, 1976; Ruiz-Lozano *et al.*, 1996; Davies *et al.*, 1993). Mycorrhiza induced drought tolerance can be related to factors influenced by AM colonization such as improved leaf water and turgor potentials (Al-Karaki, 1998; Sanchez-Blanco *et al.*, 2004), maintenance of stomatal functioning and transpiration (Estrada-Luna and Davies, 2003; Sanchez Blanco *et al.*, 2004), greater hydraulic conductivities and root development (Sivaprasad and Sulochana, 2004). AMF treated plants fully recovered plant photosynthetic activity under drought stress (Borrowska, 2002; Sivaprasad and Sulochana, 2004). Mycorrhizal colonization was found to improve water use efficiency and plant yield in watermelon (Kaya *et al.*, 2003). Christopher and Tony (2008) reported that AM symbiosis can potentially improve maize drought tolerance and the potential of this symbiotic relationship serve as an effective tool for improving cultivar drought tolerance.

2.5.2.4 Well developed root system

Due to endomycorrhizal inoculation, host plant roots have a changed morphology by increasing lateral root number and root length (Schellenbaum *et al.,* 1991). AMF inoculation stimulates rooting and enhances root production of plants, which results in better uptake of soil nutrients (Berta *et al.,* 1990; Anandaraj and Sarma, 1994a; Thanuja *et al.,* 2002). Guillemin *et al.* (1994) showed that AMF inoculation resulted in larger and more efficient root system in micropropagated plants of pineapple. AMF colonized micropropagated sugarcane plants showed excessive root growth and root dry weight, which results in better survival of these plants (Gosal *et al.,* 2001).

2.5.2.5 Salt and heavy metal tolerance

AMF can accelerate the revegetation of severely degraded lands such as coal mines or waste sites containing high levels of heavy metals (Marx, 1975). AMF association reduces impact of environmental stresses such as salinity (Ruiz-Lozano *et al.,* 1996). Mycorrhizae were found to ameliorate the toxicity of trace metals in polluted soils (Jamal *et al.,* 2002). According to Liao *et al.* (2003), *Glomus caledonicum* seems to be a promising mycorrhizal fungus for bioremediation of heavy metal contaminated soils. AM associations are reported to be present on the roots of plants growing on heavy metal contaminated soils and play an important role in metal tolerance and accumulation and isolation of these indigenous and presumably stress-adapted AMF can be a potential biotechnological tool for inoculation of plants for successful restoration of degraded ecosystems (Gaur and Adholeya, 2004).

2.5.2.6 Improved soil structure

AM hyphae play an important role in erosion control by binding soil particles together and thus maintaining soil stability (Miller and Jastrow,

1990). AMF enhances revegetation of degraded soils such as mine soils (Pfleger *et al.*, 1994) by soil stabilizing (Bethlenfalvay and Newton, 1991) and plant growth promoting effects (Comprubi *et al.*, 1990) of AM symbiosis. Rao and Tak (2002) observed that soil inoculation with *G. mosseae* has significantly enhanced plant growth and biomass production in limestone mine spoils. AM fungi improve soil texture by binding soil particles into stable aggregates that resist wind and water erosion (Rillig and Setinberg, 2002; Steinberg and Rillig, 2003).

2.5.3 Physiological and biochemical changes due to AMF colonization in host plant

Sutter (1988) studied stomatal and cuticular water loss during acclimatization in apple, cherry and sweet gum plantlets and found that in acclimatized plantlets stomatal conductance of persistent leaves decreased to about half of that in the *in vitro* leaves while cuticular conductance remained the same. He concluded that increased stomatal closure reduced the conductance. The capability of *in vitro* stomata to adapt to the new environmental conditions by modifying guard cells during acclimatization enlighten the role of stomatal closure. The capability of *in vitro* stomata to adapt to the new environmental conditions by modifying guard cells during acclimatization enlighten the role of stomata in death of micropropagated *Prunus cerasus* plants after their transfer to the external environment (Martin *et al.*, 1988). Mycorrhizal pepper plants show high net photosynthetic flux, tissue P concentration, stomatal conductance or leaf turgor during high environmental stress or recovery from stress, which indicated superior drought resistance of these plants (Davies *et al.*, 1993). The stomatal conductance was increased by *Glomus fasciculatum* in unimproved genotype of maize (Aguilera-Gomez *et al.*, 1998). The mycorrhizal plants were more water use efficient than non-mycorrhizal plants (Al-Karaki, 1998). According to Hernandez-Sebastia *et al.* (1999), root colonization by *Glomus intraradices* modifies the water status, control of water losses and osmotic relations of micropropagagted strawberry plantlets under *in vitro* conditions of high humidity.

Endomycorrhizal *(Glomus intrardices)* colonization in Chile ancho pepper alleviated low P effects by increasing internal CO_2 concentration (Aguilera–Gomez *et al.,* 1999). Micropropagated banana plants when inoculated with AMF during acclimatization phase show increased nutrient levels, photosynthesis and transpiration rates, water potential and stomatal conductance (Yano-Melo *et al.,* 1999). AMF colonized Chile ancho pepper plantlets during peak plant dehydration (Estrada-Luna and Davies, 2003). Under drought stress, mycorrhizal *Olea europaea* seedlings showed significantly higher photosynthetic and transpiration rates, stomatal conductance and foliar P concentration, than its similarly sized non-mycorrhizal counterpart (Caravaca *et al.,* 2003). Higher photosynthetic rate and stomatal conductance as early as days five and seven after inoculation with AMF than non-mycorrhizal plantlets were noticed in Chile ancho pepper (Estrada-Luna and Davies, 2003). *Rosmarinus officinalis* plants when inoculated with *Glomus deserticola* improved photosynthetic activity and stomatal conductance under water stress when compared to non-mycorrhizal stressed plants (Sanchez-Blanco *et al.,* 2004). The photosynthetic activity of plants is improved due to AMF colonization (Sivaprasad and Rai, 1985; Yano-Melo *et al.,* 1999; Dell'Amico *et al.,* 2002; Caravaca *et al.,* 2003; Sanchez-Blanco *et al.,* 2004). The reduction in stomatal and mesophyll resistance to carbon dioxide uptake, increased chlorophyll content and better hydration of plants brought about by AMF colonization favour CO_2 fixation (Sivprasad and Sulochana, 2004). Root colonization by AMF results in increased vegetative growth, total chlorophyll content and uptake of nutrients by host plant (Mathur and Vyas, 1999; Sanchez-Blanco *et al.,* 2004).

Phenolic compounds in plants were considered to be preformed inhibitors of pathogens, which play a significant role in disease resistance (Mahadevan, 1970). According to Bhatia *et al.* (1972), resistance to pathogens has been correlated with the phenol content of roots. The mycorrhizal inoculation has been reported to impart resistance to the host against a spectrum of diseases (Dehne and Schonbeck, 1979). Nemec and Meridith (1981) showed abundance of

phenols especially orthodihydric (OD) phenols in AMF structures. Mycorrhiza treated plantlets showed increased resistance to soil borne pathogens due to higher production of phenolic compounds including phytoalexins within the plant system (Hussey and Rancodori, 1982). Inoculation of peanuts with *G. fasciculatum* conferred resistance against *Sclerotium rolfsii* attack, which was related to the higher phenolic content in the host tissue (Krishna and Bagyaraj, 1986). A continuous increase of total soluble phenols in arbuscular mycorrhizal roots of *Arachis hypogea* was also reported by Krishna and Bagyaraj (1986). Histochemical studies on mycorrhizal plants showed enhanced phenolic accumulation on groundnut roots at early stages of mycorrhiza formation (Krishna and Bagyaraj, 1986). Sivaprasad and Rai (1985) reported higher activity of phytohormones like cytokinin and indole acetic acid in plants inoculated with AMF, which results in better growth and development. Increased phytohormone activity due to AMF was also reported by Allen *et al.* (1980), Barea and Azcon Aguilar (1982), Danneberg *et al.* (1992) and Goicoechea *et al.* (1995).

Mycorrhial inoculation resulted in a significant enhancement of percentage of colonization, dry matter and phenolic content of sesame plants in sterilized soils and this could be due to contribution of AM fungal structures (Selvaraj and Subramanian, 1990). Grandmaizon *et al.* (1993) found no qualitative difference in the soluble and bound phenolics isolated from non-mycorrhizal *Allium cepa* roots with those from mycorrhizal roots but the mycorrhizal roots showed higher concentration of wall bound phenolic compounds. Binding of phenolic compounds to cell wall could be responsible for the resistance of AM roots to pathogenic fungi, as it results in increased resistance by the cell wall to the action of digestive enzymes. Increased total phenol content in maize plants due to *G. fasciculatum* inoculation has been reported (Chabra and Jalali, 1995). The total phenol and orthodihydroxy phenol content of tissue culture plantlets of jack was significantly increased due to AMF inoculation (Sivaprasad *et al.,* 1995 a). High phenol content in black pepper plants was also reported by Sivaprasad *et al.* (2000). AMF colonization alleviated the physiological effects of

soil phosphate deficiency and induced changes in root phenolics and then regulated the degree of mycorrhizal colonization (Ganz *et al.,* 2002).

Higher rates of amino acids and reducing sugars in the plant root exudates was correlated with enhanced AMF colonization resulting in subsequent disease suppression (Graham *et al.,* 1981). Synthesis of new proteins or lytic enzymes in pea tissues on inoculation with mycorrhizal fungi hydrolyzed the polymers of cell wall and was considered as a defense response to invasion by parasitic organisms (Mauch *et al.,* 1988). According to Rao and Rao (1998), total soluble sugar concentration was higher in mycorrhizal plants of black gram and green gram than non-mycorrhizal plants. Boucher *et al.* (1999) showed that soluble protein concentration was remarkably increased with *Glomus etunicatum* colonization in maize plants. In clover seedlings under salt stress conditions, the intensity of salt stress showed a positive correlation with sugar accumulation for the plants inoculated with AMF (Khaled *et al.,* 2003). Nodulated clover seedlings inoculated with AMF showed antagonisitc interaction while in seedlings inoculated with AMF alone, phosphate nutrition was improved which help in salt tolerance. Both types of symbiosis helped to increase leaf protein content (Khaled *et al.,* 2003).

2.5.4 Integration of AMF technology with tissue culture

Studies have proved that successful hardening and *ex vitro* establishment of micro propagated plantlets could be achieved by inoculation with AMF at the time of planting out (Lovato *et al.,* 1996). One of the earliest reports by Granger *et al.* (1983) on the effect of AMF *(Glomus epigaeum)* on apple clones suggested that the growth and leaf mineral content of two apple clones propagated *in vitro* were increased substantially with AMF inoculation. The growth of *in vitro* cultured strawberry plants were also enhanced due to the association of AMF (Kiernan *et al.,* 1984*)*. The rooting of plantlets regenerated from callus was significantly enhanced by inoculation with *Glomus mosseae*

(Fogher *et al.,* 1986). The transplanting success and growth of *Robus idaeus* plantlets due to AMF inoculation was also reported by Pierik (1987). Mycorrhizal establishment significantly improved establishment, growth and mineral nutrition of oil palm plantlets (Blal and Gianinazzi-Pearson, 1988). The effect of AMF inoculation on micropropagated *Populus deltoids* was evaluated and it was found that rooted and hardened plants were best suited for AMF inoculation than other stages and also a positive response and colonization was obtained in terms of active root growth (Adholeya and Cheema, 1990). Micropropagated jack plantlets showed increased growth, survival rate of 80-100 per cent and increased uptake of all the elements except iron as a result of AMF association (Ramesh, 1990). Micropropagated banana plantlets inoculated with *Glomus mosseae* or *Glomus monosporum* enahanced plant growth, P and N uptake and biomass production (Rizzardi, 1990). According to Sreelatha (1992) AMF association enhanced survival, growth characteristics and uptake of nutrients in micropropagated anthurium. Inoculation with AMF seems to the key factor for subsequent growth and development of micropropagated plantlets of avocado (Vidal *et al.,* 1992). Attempt to establish rose plantlets using AMF inoculation was met with considerable success (Wilson, 1993). Inoculation of micropropagated banana plantlets with *Glomus mosseae* and *G. geosporum* resulted in greater fresh and dry weights of shoots and higher P and content (Declerck *et al.,* 1994). *In vitro* propagated cherry plants showed better growth and biomass production due to inoculation with AMF (Lovato *et al.,* 1996). Sivaprasad *et al.* (1995a) observed enhanced survival rate, growth and phenol activity in AMF inoculated jackfruit plantlets. Inoculation with *Glomus deserticola* on micropropagated plantlets of cassava in the post vitro, weaning stage enhanced per cent survival, tolerance to transplanting stress and shoot, root and tuber development (Azcon-Aguilar *et al.,* 1992). *Glomus constrictum* significantly improved the biomass production, nutrient uptake and acclimatization of *in vitro* plantlets of *Zizyphus mauritian* into the field (Mathur and Vyas, 1999). Banana plantlets inoculated with mycorrhizae during the weaning stage of micropropagation produced a more efficient root system for the uptake of phosphates and other nutrients (Severn-Ellis, 1999).

Application of *Glomus etunicatum* on micropropagated *Baptisia linctoria* promoted plant survival which is of particular interest in the propagation of pharmaceutically important clones (BK 36, BK 37) as the higher survival rates would make commercial plant production economically feasible (Grotkass *et al.,* 2000). Micropropagated *Allium sativum* plantlets inoculated with *Glomus mosseae* exhibited better growth than control plantlets under *ex vitro* conditions (Lubraco *et al.,* 2000). Inoculation on tissue culture plantlets of banana showed that AM colonization significantly increased the establishment rate, growth, vigour and biomass production during acclimatization and after transplanting to pots along with remarkable increase in P and Zn content to *G. fasciculatum* colonization (Sivaprasad *et al.,* 1999b). A significant increase in growth, vigour and biomass production during acclimatization and after transplanting to pots was observed in tissue culture plantlets of alocasia when inoculated with *Glomus* sp. and *G. fasciculatum* (Sivaprasad *et al.,* 1999a). Yano-Melo *et al.* (1999) also found that micropropagated banana plantlets inoculated with AMF had greater height, leaf area and fresh weight of shoots and roots, as well as higher rates of photosynthesis and transpiration than control plantlets. AMF inoculation increased the number of runners and daughter plants in micropropagated strawberry plants than control plants along with significant increase in N and P content in daughter plants (Alarcon *et al.,* 2000). Inoculation with *Glomus manihotis* significantly increased plant growth, root growth and nutrition of micropropagated cassava plants and also increased the field adaptability of plants, especially in marginal soils (Calderan *et al.,* 2001). According to Gosal *et al.* (2001), AMF infected sugarcane plants had better survival and excessive root growth upon transfer to soil. Taylor and Harrier (2001) reported that AMF improves mineral nutrition of micropropagated strawberry plants. Under drought conditions, AMF treated micropropagated plantlets of strawberry fully recovered their photosynthetic activity when watering was restored when compared to non inoculated plants (Borrowska, 2002). Plantlets of coffee inoculated with *Glomus clarum* increased plant height, leaf pair number, root number and also exhibited significant difference in enzymatic activities than uninoculated plants (Fernandez

et al., 2002). Cultivars of micropropagated banana showed high relative mycorrihizal dependency (RMD) values and also high N, P and K contents (Jaizme-Vega *et al.* 2002). Micropropagated taro plants after inoculation with AMF showed increased survival rate, contents of N, P, K, Cu and Zn in tissue of roots and leaves (Li *et al.,* 2002). Zeleznlik *et al.* (2002) observed that transfer of micropropagated plantlets of yellow gentian into sterile substrate with mycorrhizal inoculum produced higher number of shoots that survived compared to transfer to sterile substrate with sterile mycorrhiza or into sterile substrate without mycorrhiza. Growth and development were faster in mycorrhiza treated *Echinacea pallida* plantlets than in non treated ones and the presence of well formed arbuscules and vesicles in infested roots in addition to 90 per cent success in the survival rate of vigorous plants indicated that mycorrhization is a valuable tool to overcome *Echinacea* acclimatization shock (Lata *et al.,* 2003). *Gigaspora margarita* promoted growth, mineral nutrition and mycorrhizal colonization of micropropagated banana plants in different stages of rooting (Lins *et al.,* 2003). Micropropagated seedlings of banana when inoculated with *Gigaspora margarita* in the acclimatization phase resulted in high mycorrhizal colonization and increased growth (Trindade *et al.,* 2003). AMF inoculated micropropagated chile ancho pepper plantlets had greater transpiration rates, photosynthetic rates leaf chlorophyll content, leaf elemental N, P and K content, leaf dry biomass, leaf area and fruit production when compared with non AMF plantlets (Estrada–Luna and Davies, 2003).

2.5.5 Inoculum production of AMF

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts and must be grown in the presence of a living host plant (Habte, 1990) or at least with the host root. An effective AM fungal inoculum should promote economically significant growth, free of pathogens and contaminants and should be economically produced and formulated with sufficient shelf life. The most common techniques used for inoculum production are soil based inoculum

production, soil less substrate based inoculum production, hydroponic and aeroponic culture (Mosse and Thompson, 1984; Jarstfer and Sylvia, 1994). The quality of AMF inoculum produced is very important in tissue culture use. Several unsuccessful attempts have been made to culture the organism under *in vitro* conditions using different media (Raman and Sambandan, 2000; Raman *et al.,* 2001). Typical AMF infections can be produced in entire plants grown axenically in agar media (Mosse, 1962). Mosse and Hepper (1975) obtained AMF infections in clover root organ cultures grown in modified White's tissue culture medium containing sucrose. Axenic culture of AMF was obtained in plants grown in agar media (Allen *et al.,* 1979), sand culture (St. John *et al.,* 1981) and in flowing solution culture (McDonald, 1981).

2.5.6 Detection of AMF in roots

DNA amplification by PCR has been proposed as a technique for AM fungus identification (Simon *et al*., 1993; Wyss and Bonfante, 1993). A portion of a small sub unit rRNA gene (rDNA) specific for AM fungi can be amplified when a taxon-specific primer (VANS1) is paired with a universal primer (NS21) (Simon *et al*., 1992a). The studies of PCR amplification for AM fungi have been carried out with spores or purified DNA from colonized leek roots (Simon *et al*., 1992a; Simon *et al*., 1992b; Simon *et al*., 1993; Wyss and Bonfante, 1993). Bonito *et al*. (1995) reported detection of AM fungus *Glomus intraradices* in roots of lettuce (*Lactuca sativa* L.), leek (*Allium porrum* L.), *Zinnia* (*Zinnia elegans* Jacq), endive (*Cichorium endiva* L.) and pepper (*Capsicum annum* L.) by AM taxon-specific primer VANSI.

2.6 SYMBIOTIC ASSOCIATION OF *BACILLUS SUBTILIS* IN CASHEW

The rhizosphere around the plant roots is colonized by a large heterogeneous population of microorganisms. The various microorganisms in this habitat interact with each other through symbiotic, associative, nutralistic or

antagonistic effects depending upon nutrient status in soil and other biotic and abiotic environmental factors. Microbial interactions with plants have been found to influence the growth and yield of different crops in ways that are beneficial, neutral or detrimental (Bohlool *et al*., 1992). Due to their plant growth promoting activities, these rhizosphere bacteria have been termed "plant growth – promoting rhizobacteria" (PGPR). The PGPR strains have great potential to be used as inoculants in the agricultural system and have received increasing attention during the last two decades. Rhizosphere bacteria (rhizobacteria) with plant growth promotion ability and pathogen suppressive abilities are being considered as potential inoculants for enhancing crop productivity (Sindhu *et al*., 1999; Compant *et al*., 2005a). Among rhizobacteria, *Bacillus* species are commonly found in rhizospheres of leguminous and non-leguminous crops. Several mechanisms have been suggested by which *Bacillus* sp. can promote plant growth (Kloepper *et al*., 2004).

2.7 Hairy root disease

Agrobacterium is a soil bacterium which produces hairy root disease which was first demonstrated by Ricker (1930). The physiological basis of hairy root disease is not totally understood. *A. rhizogenes* is a Gram (-) bacterium commonly present in soils. It has the ability to infect plants through wounds and as a result, it induces abundant adventitious roots (Tepfer, 1984; Petit *et al*., 1986). In fact, rooting of *Pinus* spp., *Larix* spp., *Eucalyptus* spp. and *Sequoia sensperv*irens was successfully achieved by using strains of this bacterium (McAfee *et al*., 1993; MacRae and Staden, 1993; Mihaljevic *et al*., 1999). *A. rhizogenes* isolates have been used in horticulture to induce roots in a difficult to root subclones of apple (Patena *et al*., 1988) and for propagation of hazelnut cuttings (Bassil *et al*., 1991). Strobel and Nachmias (1985) have observed increased root numbers and root mass in almond trees that had been treated with suspensions of *A. rhizogenes*. Similarly improved growth and yield has been

reported for olive trees when the roots were inoculated with this bacterium (Strobel *et al.*, 1988).

2.7.1 Mechanism of *Agrobacterium* plant cell interaction

One of the earliest stages in the interaction between *Agrobacterium* and a plant is the attachment of the bacterium to the surface of the plant cell. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds such as acetosyringone, that activate the vir region of the bacterial plasmid (Binns and Thomashow, 1988).

The morphogenic effects have been attributed to the transfer of part of a large plasmid known as the Ri (root inducing) plasmid. The symptoms observed with *A. rhizogenes* are suggestive of auxin effects resulting from an increase in cellular auxin sensitivity rather than auxin production. Shen *et al.* (1988) found that the sensitivity of the tips of Ri-induced roots to exogenous auxin was 100-1000 times higher them that of uninduced roots.

When plant tissues are transformed by the Ri plasmid, a specific DNA sequence called T-DNA are transferred to the plant nuclear genome. This DNA is divided into TL (left) and TR (right) regions in agropine-type Ri plasmids. Four TL-DNA loci affecting hairy root induction have been identified (White *et al.*, 1985) but their specific functions are only partly understood (Schmulling *et al.*, 1988). The auxin synthesis genes are found in the TR-DNA (White *et al.*, 1985) and have been shown to play an accessory role in root induction by providing the auxin needed to trigger differentiation of auxin responsive transformed cells. On other hand, *A. rhizogenes* devoid of T-DNA born auxin genes is incapable of eliciting symptoms in the absence of IAA, indicating the necessity of auxin for hairy root induction (Cardarelli *et al.*, 1987). Another consequence of the transfer of the T-DNA into the plant genome is the diversion of plant metabolites into the production of opines that in turn can be

catabolized by the bacterium. Synthesis of opines is generally recognized as evidence that transformation by *Agrobacterium* has occurred (Hooykaas-Slogteren *et al.*, 1984; Hernalsteens *et al.*, 1984).

2.7.2 Genes responsible for root induction

Root formation is due to the integration of pRi T-DNA into the plant genome and its subsequent expression (Chilton *et al.*, 1982). Genes harboured by the root inducing (Ri) plasmid could be used to transform woody species and to induce adventitious rooting in their explants. Also root transformation using the Ri plasmid may result in a more effective root system (Torrey, 1988).

The Ri plasmids are large (200 to greater than 800 Kb) and contain one or two regions of T-DNA and a vir (virulence) region (Gelvin, 1990). Each of the T-DNA fragments spans a 15-20 Kb region, and they are separated from each other by atleast 15 Kb of non integrated plasmid DNA.

The genes encoding auxin synthesis (tms 1 and tms 2) and agropine synthesis (ags) have been localized on the TR-DNA of the agropine type Ri plasmid (Cardarelli *et al.*, 1985). TL-DNA plays the major role in hairy root induction and the genes encoding auxin synthesis have accessory role (Palazon *et al.*, 1997).

The soil bacterium *A. rhizogenes* induces a proliferation at the site of infection (Elliot, 1951). This is due to the transfer to the host cells of a portion of Ri plasmids (Root inducing) T-DNA (Chilton *et al.*, 1982; White *et al.*, 1982; Willmitzer *et al.*, 1982).

Materials and Methods

3. MATERIALS AND METHODS

The study on management of recalcitrancy in *in vitro* cultures of cashew (*Anacardium occidentale* L.) was carried out the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period of 2006 to 2008. Description of the materials used and methodology adopted in the study have been furnished in this chapter.

3.1 MATERIALS

3.1.1 Chemical, glassware and plastic ware

The chemicals used for the study were of good quality (AR grade) from various agencies including MERCK, SRL, SIGMA and HIMEDIA. Molecular Biology grade enzymes and buffers were supplied by Bangalore Genei LTD. All the plasticware used was obtained from Axygen and Tarson India LTD. The primers were obtained from Carmina Renteria, Labware Scientific, INC, USA. Pure culture of VAM was obtained from College of Agriculture, Vellayani and UAS, Bangalore.

Borosilicate glassware of Corning/Borosil/brand and disposable pertridishes of Axygen and Tarson were used for the present study. The glassware were initially subjected to steam and cleaned with detergent solution and then with tap water. The glassware was then soaked in a solution containing potassium dichromate, water and sulphuric acid (8:80:250) followed by thorough washing with jets of tap water and finally with distilled water. Test tubes were kept in racks and dried in open air. Conical flasks were dried in the hot air oven. Jam bottles were further sterilized in an autoclave and dried in the hot air oven. The centrifuge tubes were first washed in soap solution and then with distilled water. The tubes were sterilized in an autoclave and kept in the hot air

oven. Glass petriplates were washed, dried in open air, wiped with alcohol. It was then autoclaved and dried in the hot air oven.

3.1.2 Equipments and machinery

The equipments available at the Centre for Plant Biotechnology and Molecular Biology and Bioinformatices centre, College of Horticulture were used for the present study. The PCR was done in thermal cycler for the model Master Cycler personal (Eppendorf) and horizontal gel electrophoresis system (Genei) was used for agarose gel electrophoresis.

3.1.3 Composition of media

The basal media used for the study is Murashige and Skoog (1962) medium (MS). Half strength MS was also used. The composition of the media are given in Annexure I.

The different *Agrobacterium rhizogenes* were cultured in Yeast Extract Broth (YEB) and Nutrient Agar (NA). Endophytic and covert microorganism and *Bacillus subtilis* were cultured in nutrient agar medium. Endophytic fungi were cultured in potato dextrose agar medium. The basal compositions of these media are given in Annexure II.

3.1.4 Preparation of tissue culture medium

Standard procedure (Gamborg *et al.*, 1968) was followed for the preparation of plant tissue culture media. Stock solutions of major and minor nutrients were prepared and stored in pre-cleaned glass bottles in refrigerated conditions. Stock 3 was stored in amber coloured bottles.

A clean steel vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. For preparing media of full strength, 20 ml was pipetted from 50x stocks and 10 ml from 100x stocks. A small volume of distilled water as added to it and later on, required quantities of sucrose and inositol were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of the medium was adjusted to 5.7 using 0.1N NaOH.

For solid medium, agar was added at 0.75 per cent (w/v) concentration after adjusting the pH. The medium was stirred and heated to melt the agar, and was poured when hot into culture vessels which were plugged with absorbent cotton. For solid media, test tubes (15 cm X 2.5 cm), (25 cm X 5 cm) were used. For liquid media, conical flasks (100, 250 and 500 ml) were used as culture vessels. Fifteen ml medium was poured in each test tube, 30 ml medium in 100 ml conical flasks. Vessels containing media were sterilized in an autoclave at 121°C in 15 psi for 20 min. The medium was allowed to cool to room temperature and stored in culture room until used.

3.1.5 Growth regulators

Auxins (2,4-D, NAA, IAA) and cytokinins (BA, Kin) were incorporated in the media at various stages of culturing for establishment, multiplication and rooting.

3.1.6 Organic supplements

L-glutamine (0.4 g l^{-1}) was tried for its effect on establishment of field grown explants.

3.1.7 Carbon source

Sucrose (3%) was used as the main source of carbon in this study.

3.1.8 Preparation of YEB, NA and LBA medium

Clean steel vessels, rinsed with distilled water were used to prepare the media. The ingredients were weighed on electronic balance and were added into the vessels. A small volume of distilled water was added to it and the ingredients were dissolved. The desired volume was made up by adding distilled water. The pH of the media was adjusted to 7.0 for NA and LBA and 7.2 for YEB using a standard pH meter by adding either 0.1N NaOH or HCl.

For solid media, agar was added at the rate of 20 gl^{-1}. The media were stirred and heated to melt the agar and were poured when hot, into conical flasks and jam bottles. Conical flasks were plugged with non-absorbent cotton and jam bottles were sealed tightly using cello tape after placing the lid. Autoclaving was done at 121°C at 15 psi for 20 min. to sterilize the medium and they were further kept in the culture room until used.

3.1.9 Antibiotics

The stock solutions of antibiotics were prepared fresh under sterile conditions. Aliquots were taken from them and were added to the sterilized media. The solid media was first melted, cooled to 40°C and then the desired quantities of aliquots of antibiotics were added to them.

Different antibiotics (ampicillin, cefotaxime, gentamycin, nalidixic acid, streptomycin, tetracycline) were used in MS medium, YEB medium, Nutrient Agar medium and LB medium for killing the bacteria, for

testing the resistance of bacteria to antibiotics and also for studying the sensitivity of explants to various antibiotics.

3.1.10 Preparation of potato dextrose agar (PDA) medium

Boiled finely sliced potatoes in 500 ml of water until thoroughly cooked; strained through cheese cloth. Ingredients were added into the vessels. The desired volume was made up by adding distilled water to filtrate. The pH of the media was adjusted to 5.6 using a standard pH meter by adding either 0.1N NaOH or HCl. Agar was added at the rate of 15 g l^{-1}. The media was stirred and heated, to melt the agar and were poured when hot into conical flasks and jam bottles.

3.2 ISOLATION AND IDENTIFICATION OF ENDOPHYTIC AND COVERT BACTERIA

3.2.1 ISOLATION OF ENDOPHYTIC BACTERIA

The procedure as reported by Dinakaran *et al* (2003) was used. Sample materials were cut into small pieces. Surface disinfection was done by treating with 20 per cent hydrogen peroxide for 10 minutes followed by 5 rinses in 0.02M potassium phosphate buffer at pH 7. To confirm the surface sterilization, 0.1 ml of final washing solution of each sample was transferred to a petri plate with nutrient agar media and incubated at 28^{o}C for 3 days. The tissue (1g) was ground in sterilized pestle and mortar in 9.9 ml of the final buffer and serial dilutions up to 10^{-3}. From each dilution, 1 ml was transferred to petri plates and poured in nutrient agar medium and mixed thoroughly. Replica for each sample was also maintained. Plates were incubated for 24 to 48 hr at 28+/- 2^{o}C. The colony count was taken and endophytic population per gram of tissue was calculated. Bacterial colonies developed were picked and transferred to nutrient agar media, purified and stored.

3.2.2 ISOLATION OF COVERT BACTERIA

Covert bacteria were isolated from stem and petiole of *in vitro* growing cashew plants in multiplication, elongation and rooting media.

3.2.2.1 Culture Indexing

The presence of bacteria in the cultures was assessed using nutrient agar, the bacteriological indexing media (BIM). The cultures were scored positive or negative for bacteria based on colony growth on BIM after 48-72 hrs and again after 1-2 weeks. The nutrient plates used for indexing were pre-incubated at 37°C for 2-3 days to ensure freedom from accidental contaminants.

3.2.2.2 Antibiotic treatment

Gentamycin and streptomycin were incorporated to multiplication, elongation and rooting media at the concentrations of 0, 10, 50, 100 mgl^{-1} and shoot bud cultures were inoculated. Response of cultures was observed and indexed for bacteria after one month. Antibiotic gentamycin (Gram negative bactericidal), streptomycin (Gram positive bactericidal) were provided as 2 ml overlay to control the overgrowth of bacteria in the media and cultures were inoculated. The cultures were indexed for any covert bacteria using nutrient agar and were observed for root and shoot growth 1 month after culturing.

3.2.2.3 Isolation of bacteria and purification

Colony growth obtained on nutrient medium after indexing of antibiotic treated cultures was used for bacterial isolation. Serial dilutions were plated on nutrient agar and after overnight incubation at 30°C, distinct single colonies were picked up.

3.2.3 Morphological characterization

Gram staining was used for the characterization of all the bacterial isolates. Chemical compositions of the reagents used for staining reaction are given in Annexure III.

3.2.3.1 Gram staining

The isolates were subjected to Gram staining reaction following the procedure of Hucker and Conn (1923).

1. A smear of the culture was prepared on a clean slide, air dried and heat fixed.
2. One to two drops of crystal violet stain was added and the smear washed after one minute.
3. Grams iodine solution was added for one minute and then washed.
4. Decolourized with ethyl alcohol for 30 seconds.
5. The smear was treated with the counter stain safranine for one minute.
6. Finally the slide was washed under running tap water, air dried and observed under 100 x objective of a compound binocular microscope.

3.2.4 INTRINSIC ANTIBIOTIC RESISTANCE (IAR) PATTERN

Nutrient agar was used to study the intrinsic antibiotic resistance pattern of endophytic and covert bacteria. To the molten medium, required concentrations of antibiotics (Table 1) were added, mixed and poured into petri plates. Each petri dish was divided into two grids and each covert bacteria was streaked in one grid. Endophytic bacteria was streaked separately on one plate. Three replications were maintained for each concentration of the antibiotic. Medium without any antibiotics served as control. Plates were observed for presence or absence of growth after 24 hr of incubation at room temperature.

Table 1. Details of antibiotics used for intrinsic antibiotic resistance screening

Sl. No	Antibiotic	Concentration of stock (%)	Solvent	Working concentration (mg ml^{-1})
1	Ampicillin	5	sterile water	25
				50
				100
2	Cefotaxime	10	sterile water	50
				100
				250
3	Gentamycin	1	sterile water	5
				10
				25
4	Nalidixic acid	1	sterile water	5
				10
				15
5	Streptomycin	10	sterile water	50
				100
				200
6	Tetracyclin	1.5	70% ethanol	10
				15
				25

3.2.5 ISOLATION OF BACTERIAL GENOMIC DNA

The procedure reported by Schleif and Wensink (1981) for isolation of DNA was tried for genomic DNA isolation in bacteria. Bacteria was inoculated in to 3ml nutrient agar broth and kept at 28°C and at 160 rpm. Twenty four hr old culture was used for isolation of bacterial DNA.

Reagents used

1. Lysozome (1mg ml^{-1})
2. 0.5 M EDTA
3. 3 M Sodium acetate
4. SDS (10%)
5. Phenol : chloroform (1:1, v/v)
6. Chloroform
7. Distilled water or TE buffer

Details of composition of reagents were provided in Annexure IV.

Procedure

- 1.5 ml of culture was centrifuged at 5000 rpm for 5 minute
- Decanted the supernatant
- Repeated the Step 1& 2
- Resuspend the pellet in 500 µl of distilled water
- 5 µl of lysozyme (1mg ml^{-1}) and 120 µl of 0.5 M EDTA (pH -8.0) was added and mixed gently and incubate at 37°C for 5 min at room temp
- 70 µl of 10 per cent SDS was added and mixed by inversion and incubate at 37°C for one hour
- 600 µl of equilibrated phenol : chloroform (1:1) was added and mixed gently by inversion

- Centrifuged at 7000 rpm for 10 min at 4°C
- Top aqueous layer was transferred to fresh tube and re-extracted as before
- 600 µl of chloroform isoamyl alcohol was added and mixed thoroughly by inversion
- Centrifuged at 8000 rpm for 10 min at 4°C
- Top aqueous layer was transferred to another fresh tube and 60 µl of 3 M sodium acetate (pH- 5.8) and 1 ml of ice cold absolute ethanol was added to precipitate the DNA
- Centrifuged at 10, 000 rpm for 5 min. The DNA pellet was washed with 70 per cent ethanol and air dried
- Resuspend the DNA pellet in 100 µl of distilled water

3.2.6 ESTIMATION OF QUANTITY OF DNA

The quality of isolated DNA was determined through agarose gel electrophoresis (Sambrook *et al*., 1989).

Materials for agrose gel electrophoresis

1) Agarose (Genei; Low EEO) - 0.7 per cent (for genomic DNA)
   - 1.2 per cent (for PCR Samples)
2) 50X TAE buffer (pH - 8.0)
3) 6X Loading / Tracking dye
4) Ethidium bromide solution (stock 10 mg ml^{-1}; working concentration: 0.5 µg ml^{-1})
5) UV transilluminator (Herolab^{R})
6) Electrophoresis, unit, power pack, gel casting tray, comb
7) Gel documentation and analysis system (UVP, GelDoc It ^{TM} Imaging system, UK)

Composition of reagents is provided in Annexure V.

Procedure for agarose gel electrophoresis

1. The 50X stock solution of TAE buffer was diluted to 1X concentration
2. The required quantity of agarose (1%) was weighed and dissolved completely in 1X TAE buffer by boiling
3. The solution was cooled to lukewarm temperature (55^{o}C) and ethidium bromide was added at a concentration of 0.5 μg ml^{-1}.
4. The open ends of the gel-casting tray were sealed using cello tape and placed on a horizontal leveled platform. The comb was placed properly and the solution of agarose was poured in to the gel casting tray
5. The gel was allowed to set for 30 to 45 minutes at room temperature. After solidification, the comb was removed carefully and the tape was pulled off the gel casting tray
6. The gel was placed in an electrophoresis unit (Genei) containing IX TAE buffer with the wells directed towards the cathode. Required quantity of IX TAE buffer was added so us to submerge the gel to a depth of I cm
7. A piece of Para film was pressed on a solid surface and 1μl 6X loading dye was dispensed in small quantity on the tape. A quantity of 3 to 5 μl of DNA was added to each slot and mixed well by pipetting in and out for 2 to 3 times
8. The samples were carefully loaded in the wells using a micropipette. The λ DNA/ *Eco* RI + *Hind* III double digest (Bangalore Genei) was loaded in one of the wells as the molecular weight marker
9. The cathode and anode of the electrophoresis unit were connected to the power supply. The power supply was turned on and the gel was run at constant voltage (100 volts) till the tracking dye reached 2/3 rd length of the gel
10. Then the current was disconnected and the gel was removed from the electrophoresis unit
11. The gel was placed in a gel documentation system (UVP, GelDoc It ^{TM} Imaging system, UK), and bands were visualized under UV light in a

transilluminator and the gel image was documented. Thus the quality of DNA extracted was ensured

3.2.7 QUANTIFICATION OF DNA

The genomic DNA isolated and detected as good through agarose gel electrophoresis were further analyzed for its quantity using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1µl autoclaved distilled water as blank. One microlitre from each sample was quantified and was measured in ng/µl. The absorbances of nucleic acids were measured at 260 nm and 280 nm. The purity of DNA was assessed by the ratio OD_{260} / OD_{280}. A ratio of 1.8 to 2.0 indicated pure DNA. The quantity of DNA in the pure sample was calculated using the formula

OD_{260} = 1 is equivalent to 50 ng double stranded DNA.

1 OD at 260 nm = 50 ng ml^{-1} DNA

Therefore OD_{260} X 50 gives the quantity of DNA in ng ml^{-1}

3.2.8 PCR (Polymerase Chain Reaction)

After isolation of good quality of genomic DNA from covert and endophytic bacteria. PCR was performed for the amplification of 16S rDNA. 16S rDNA gene of isolates was amplified using two universal bacterial primers: $16S_{43-63}$ (5'-CAGGCCTAACACATGCAAGTC-3') and $16S_{1404-1387}$ (5'-GGGCGGWGTGTACAAGGC-3') (Radeva and Selenska-Pobell, 2005). PCR products were separated on 1 per cent agarose gel.

PCR reaction mixture contains different constituents like template DNA, universal bacterial primer enzymes, dNTPs, $MgCl_2$ and assay buffer which are subjected to repeated cycles of denaturation; primer annealing and extension in thermal cycles.

3.2.8.1 Protocol for PCR

The basic PCR procedure suggested by Mullis (1986) was suitably modified to carry out 16S rDNA amplification.

Initial denaturation - 95°C for 3 minutes
Denaturation - 94°C for 90 seconds
Primer annealing - 55°C for 40 seconds } 35 cycles.
Primer extension - 72°C for 90 seconds
Final extension - 72°C for 20 minutes
4^0C for infinity to hold the sample.

PCR amplification was performed in a 25 µl reaction and each reaction had the following compounds.

Table 2. Preparation of PCR master mix

Components	**Vol. per reaction (µl)**
Genomic DNA (25ng)	1.0
10 X *Taq* assay buffer containing $MgCl_2$	2.5
dNTP mix (10 mM each)	1.0
Forward primer	1.0
Reverse primer	1.0
Taq DNA polymerase (0.5u)	2.0
Autoclaved distilled water	16.5
Total volume	**25.0**

Master mix was prepared for the required number of reactions adding all the components of the reaction mix except the primer. Aliquots of the master mix (24 µl) was pipetted out into each of the 0.2 ml PCR tubes placed on

ice followed by addition of 1µl DNA into each tube separately. A brief spinning was given for the reaction, the tubes were loaded in the thermal cycler and the PCR programme was run.

The PCR products were resolved on 1 per cent agarose using 1X TAE buffer stained with ethidium bromide. 16S rDNA amplifications were visualized under UV (312 nm) transilluminator and documented using gel documentation system (UVP, GelDoc It ™ Imaging system, UK).

3.2.9 MOLECULAR CLONING

3.2.9.1 Gel elution of specific amplicon

Products obtained in PCR reactions were loaded separately on 1.0 per cent (w/v) agarose gel and amplified band in each bacteria was eluted using Axy Prep DNA Gel Extraction Kit (Axygen, Bioscences). Procedure as per the manufacturer's guidelines was followed.

- DNA fragment of interest was excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on a transilluminator.
- Gel slice was weighed in a colourless 1.5 ml microfuge tube.
- Added 3X gel volume of gel solubilization buffer (w/v)
- The gel was resuspended in gel solubilization buffer by vortexing. Heated the gel containing gel solubilization buffer at 75°C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 minutes to accelerate gel solubilization.
- Added 0.5X gel solubilization volume of binding buffer and mixed properly.
- A spin column was placed in a 2 ml collection tube. The solubilized gel slice was transferred into the spin column that was assembled in the 2ml collection tube and centrifuged at 12, 000g for one minute.

- The filtrate was discarded. Added 500 µl of wash buffer to the spin column and centrifuged at 12,000g for 30 seconds.
- Discarded the filtrate and 700 µl of desalting buffer was added and centrifuged at 12,000g for 30 seconds.
- A second wash, by adding 700 µl of desalting buffer followed by centrifugation at 12,000 rpm for 30 seconds was done to ensure the complete removal of salt.
- The filtrate was discarded and spin column was again placed in collection tube.
- Column was again centrifuged for 1 minute at 12,000 rpm to remove any residual buffer.
- Spin column was transferred to a fresh 1.5 ml centrifuge tube. The eluent was prewarmed at 65°C to improve the elution efficiency. To elute the DNA, 25 µl of eluent was added to the centre of the spin column. It was allowed to stand for one minute at room temperature. Then centrifuged at 12,000g for one minute.
- Eluted DNA fragments were checked on 0.8 per cent (w/v) agarose gel and stored at 20° C for further cloning work.

3.2.10 TRANSFORMATION

3.2.10.1 Preparation of competent cells

Competent cells for plasmid transformation were prepared following the protocol of Mandel and Higa (1970). Medium prepared: LB medium and LBA medium (Details of media are given in the Annexure II).

The steps followed for competent cell preparation were as follows:

Day 1:

Inoculated 18 hrs old *Escherichia coli* JM 109 strain, single colony to 3ml LB medium in sterile condition and incubated overnight at 37°C on a shaker set at 160 rpm.

Day 2:

1. Aseptically transferred 3 ml overnight grown culture to 50 ml sterile LB broth and inoculated for 4 hr at 37°C on a shaker set at 160 rpm until OD_{600} reached 0.4 to 0.5. The growth of culture was monitored at every 30 minutes
2. The cells were aseptically transferred to a sterile disposable ice-cold 50 ml polypropylene tube
3. The culture was centrifuged at 3500 rpm for 10 minutes at 4°C
4. The supernatant was carefully discarded and the pellet was gently resuspended in 10 ml ice- cold 0.1 M $CaCl_2$
5. The tubes were kept on ice for 20 minutes and the cell suspension was centrifuged at 5000 rpm for 10 minutes at 4°C
6. The supernatant was decanted and the pellet resuspended in 2 ml of ice cold filter sterilized 0.1 M $CaCl_2$
7. The tubes were kept on ice for 18 hours

Day 3:

1. Chilled glycerol (4 ml) was added to the cell suspension and mixed well using a sterile micro tip
2. The competent cells prepared were stored at -70°C as aliquots of 100 µl in chilled 1.5 ml microfuge tubes covered with aluminum foil until further use

3.2.10.2 Screening of competent cells

Transformation of competent cells with a plasmid having ampicillin resistance (pUC 18) was carried out to check the competence and purity of competent cells. The procedure followed for screening of plasmid is as follows.

1. The competent cells stored at -70°C were thawed over ice for 10 minutes
2. Plasmid (10 µl) was added to 100 µl competent cells. Negative control was placed simultaneously without adding plasmid
3. The cells were kept over ice for 40 minutes. Heat shock was given at 42°C for 5 minutes
4. LB medium (250 µl) was added to the cells and incubated at 37°C for I hour on a shaker set at 120 rpm
5. The transformed cells (100 µl) were plated on LBA medium containing ampicillin and incubated overnight at 37°C in a shaker (100 rpm). The recombinant clone alone can grow on ampicillin plate

3.2.11 CLONING OF ELUTED DNA

The eluted product was cloned in pGEM-T vector (Fig.1) using pGEM-T Easy vector system supplied by Promega, USA.

3.2.11.1 Ligation

The pGEM-T Easy vector was centrifuged briefly to collect the contents at the bottom of the tube. Ligation reaction was set up in 0.5 ml microfuge tubes as follows.

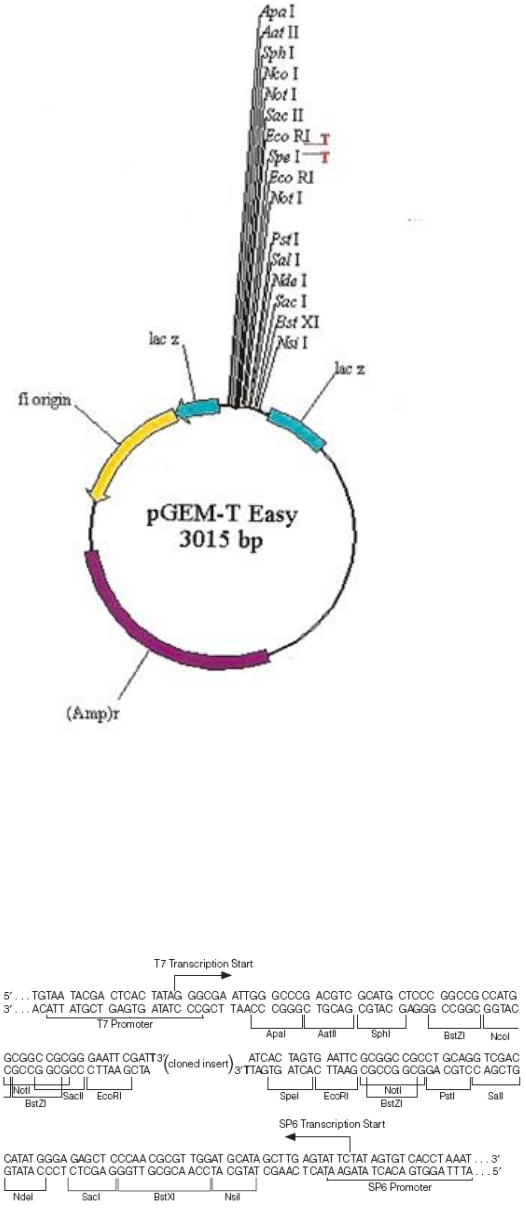


Fig. 1. pGEM-T Easy Vector (Promega) used for cloning PCR product.

The *lac*Z region, promoter and multiple cloning sites are shown in the figure. The top strands of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase and bottom strands corresponds to the RNA synthesized by SP6 RNA polymerase.

Ingredients	Standard Reaction	Positive control	Background control
Rapid ligation buffer (2x)	5 µl	5µl	5µl
pGEM-T Easy Vector (50 ng)	1 µl	1µl	1µl
Eluted DNA	1µl	-	-
Control insert DNA	-	2µl	-
T_4 DNA ligase (3 Weiss units 2µl)	1µl	1µl	1µl
Deionized water	2µl	1µl	3µl
Final volume	10µl	10µl	10µl

The reaction mix was mixed and incubated for 1 hour at room temperature and then incubated at 4°C overnight. It was used on the next day for transformation in competent cells of *E. coli.*

3.2.11.2 Transformation of ligated product and recombinant selection

Reagents prepared:

1. Ampicillin - 5 mg ml^{-1} in water
2. IPTG - 200 mg ml^{-1} in water
3. X-gal - 10 mg ml^{-1} in DMSO

The procedure followed for DNA transformation and blue white screening is as follows.

The ligated PCR product was added to 100 µl of competent cells and kept on ice for 40 minutes. Heat shock was given at 42°C for 2 minutes in a water bath and plunged in ice for 5 minutes. LB medium (250 µl) was added to the cells and incubated at 37°C for 1 hour on a shaker set at 120 rpm. The aliquots

of cells (100 µl) were plated on LBA / ampicillin (5mg μl^{-1}) / X-gal (20 mg ml^{-1}) plates and incubated overnight at 37°C.

3.2.11.3 Isolation of recombinant plasmid DNA

Plasmid DNA was isolated from white colonies using alkaline mini prep procedure as described by Birnboim and Doly (1979).

Reagents prepared

Resuspension buffer (solution I)
Lysis buffer (solution II)
Neutralization buffer (solution III)
LB medium
Ampicillin (50 mg l^{-1})

(Chemical composition of reagents are given in Annexure VI)

Procedure

1. Cells were harvested from overnight grown recombinant *E. coli* culture from white colonies containing plasmid DNA by centrifugation at 10, 000 rpm for 5 minutes
2. The supernatant was discarded and the bacterial pellet was suspended in ice-cold resuspension buffer (100 µl)
3. The freshly prepared lysis buffer (200 µl) was added to the pellet and mixed gently by inverting the tubes for 5 minutes
4. Ice-cold neutralization buffer (150 µl) was added, vortexed gently and kept on ice for 5 minutes
5. Centrifuged at 12,000g for 10 minutes at 4^0C and the supernatant was transferred to a fresh Eppendorf tube

6. The DNA was precipitated with two volume of ethanol at room temperature and vortexed
7. Incubated at room temperature for 2 minutes and centrifuged at 12,000g for 5 minutes at 4^0C
8. The supernatant was removed and pellet was rinsed with 70 per cent ethanol
9. The pellet was air dried and resuspended in 30 µl autoclaved double distilled water
10. Five microlitre of the plasmid suspension was checked on agarose gel and the image was documented

3.2.12 CONFIRMATION OF DNA CLONES

3.2.12.1 Confirmation of recombinant plasmid using PCR

The recombinant plasmid DNA isolated by alkali lysis method was amplified by PCR (Eppendorf Master Cycler Personnel) in 25 µl reaction mix using M13 primer. The following PCR template was prepared.

1. Diluted 1µl of plasmid DNA (≈25 ng) to 10 µl with sterile H_2O.
2. One microlitre of diluted plasmid DNA was added in a new PCR tube.
3. A Master Mix was prepared and the reagents used were in the sequential order as shown in the Table 3.
4. The contents were mixed well and briefly centrifuged.
5. Twenty four micro litres of Master Mix was aliquotted into 0.2 ml microfuge tube.

The following PCR programme was run immediately.

Step1. Initial denaturation at 95°C for 3 minutes

Step 2. Denaturation at 94°C for 90 seconds
Step 3. Primer Annealing at 55°C for 40 seconds } 35 cycles
Step 4. Primer extension at 72°C for 90 seconds

Step 5. Final extension at 72°C for 20 minutes

Table 3. Preparation of PCR master mix

Components	Vol. per reaction (µl)
Genomic DNA (25 ng)	1.0
10 X *Taq* assay buffer containing $MgCl_2$	2.5
d NTP mix (10 mM each)	1.0
M13 Forward primer	1.0
M13 Reverse primer	1.0
Taq DNA polymerase (0.5u)	2.0
Autoclaved distilled water	16.5
Total volume	**25.0**

A momentary spinning was given for the reaction and set in thermal cycles for polymerase chain amplification with the above mentioned programme. The PCR product was checked on 1 per cent agarose gel and documented.

3.2.13 MAINTENANCE OF CLONES

3.2.13.1 Preparation of pure culture of recombinant bacteria

Materials prepared

LBA medium with ampicillin (5 %)

In a laminar flow, single white colony from the transformed plate was taken by using flame sterilized bacterial loop. This was streaked on LBA plate containing ampicillin (50 mg l^{-1}). The plate was incubated overnight at 37°C and further stored at 4°C.

3.2.13.2 Preparation of stabs

Materials prepared

LBA medium with ampicillin (5 %)

The LBA medium containing the antibiotic ampicillin (50 mg l^{-1}) was melted and poured in to a storage vial, aseptically and allowed to solidify. Single colony of recombinant bacterial colony was carefully lifted with a sterile bacterial loop. The loop loaded with bacteria was plunged in to the solid media and incubated at 37°C overnight in the culture tube. The stabs showing good growth of bacteria were further stored in refrigerator at 4°C.

3.2.13.3 Glycerol culture

Materials prepared

LB liquid with ampicillin (5 %)

In a laminar air flow, recombinant colony from the transformed plate was taken using a flame sterilized loop. This was plunged in to LB medium containing the antibiotic, ampicillin (50 mg l^{-1}). It was incubated at 37°C in a shaker at 120 rpm overnight. On the next day aliquots (800 µl) of cell culture was added to 100 per cent glycerol (200 µl) aseptically and stored at -20^0C.

3.2.14 SEQUENCE OF DNA CLONES

The stab of the recombinant clone of three bacteria were sent to DNA sequencing facility, Bangalore Genei (www.Bangaloregeni.com) for sequencing. Details of the vector cloned and size of the insert were provided. Sequencing was done with SP6 primer to obtain 5'- 3' sequence information of the insert from the reverse region, using automated sequencer, ABI-31100 Genetic Analyzer, that used fluorescent labeled dye terminators and fluorescent labeled primers. The Sanger's method of sequencing was reported to be adopted by the firm.

3.2.15 THEORETICAL ANALYSIS OF SEQUENCE

The sequence information of KAU-EC1, KAU-CC1 and KAU-CC2 obtained from the firm was further analyzed for its characterization.

3.2.16 VECTOR SCREENING

To remove the vector regions present in the sequences, vector screening was performed using VecScreen tool (www..ncbi.nlm.nih.gov /VecScreen) provided by NCBI. The vector and the adaptor sequences present were removed using Bioedit- Biological sequence alignment editor tool.

3.2.17 ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI

Fungal species were isolated from 2, 4 and 6 weeks after culturing of nodal segments derived from field plants. Fungal species were purified by inoculating into PDA media and sent to the National Centre of Fungal Taxonomy, New Delhi for identification of fungi.

3.2.17.1 MANAGEMENT OF ENDOPHYTIC FUNGI

Spraying with 0.1 per cent Bavistin onto field explants and treatment with 0.1% Bavistin for 15 min. before surface sterilization of explants. $CuSO_4$ at 100, 150 and 200 mg l^{-1} was added into establishment media. Bavistin at 0.025, 0.05 and 0.1% was also added into different media.

3.3 ESTIMATION OF TOTAL PHENOLS

Phenol leached into the media was determined by inoculation of the nodal segments into sterile water and liquid media after washing with running tap water for 30 min. Alcoholic extracts of nodal segments from young trees (7-8 years) were also taken for the estimation. The Folin-Ciocalteau method (Malick and Singh, 1980) was followed for the estimation. Phenol reacts with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Reagents required

1. Ethanol (80%)
2. Folin-Ciocalteau reagent (1:100)
3. Na_2CO_3 (20%)
4. Standard (100 mg Catechol in 100 ml water)
   Dilute 10 times for a working standard

Procedure

1. Weigh 1g of the sample and grind it with a pestle and mortar in 10-time volume of 80 per cent ethanol

2. Centrifuge homogenate at 10,000 rpm for 20 min. Save the supernatant. Re-extract the residue with five times the volume of 80 per cent ethanol, centrifuge and pool the supernatants
3. Evaporate supernatant to dryness
4. Dissolve the residue in a known volume of distilled water (5 ml)
5. Pipette out different aliquots (0.2 to 2 ml) into test tubes
6. Make up the volume in each tube to 3 ml with water
7. Add 0.5 ml of Folin-Ciocalteau reagent
8. After 3 min, add 2 ml of 20 per cent Na_2CO_3 solution to each tube
9. Mix thoroughly. Place the tubes in a boiling water for exactly one min, cool and measure the absorbance at 650 nm against a reagent blank
10. Prepare a standard curve using different concentrations of catechol

Concentration of phenol in the sample was calculated by the following formula.

Total phenol content present in the sample =

$$\frac{\text{OD of phenol X total phenol X 1 X std. conc.}}{\text{OD of standard X volume taken X sample weight}}$$

3.4 TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic manipulations such as surface sterilization of explant preparation and inoculation of explants, subsequent sub culturing, preparation of antibiotic media, cloning and transformation work were carried out in a laminar airflow cabinet.

3.5 CULTURE ROOM

The cultures were incubated at 26 ± 2°C in an air-conditioned culture room with 16 hrs light photoperiod (1000 lux) from fluorescent tubes.

Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing. Dark condition for culturing bacteria was provided by black cotton cloth fixed on culture racks.

3.6 SOURCE OF EXPLANTS

In vitro seedlings and nodal segments from field grown young cashew trees (7- 8 years) were the two sources of explants.

3.7 STANDARDIZATION OF *IN VITRO* REGENERATION

3.7.1 Explants used for micropropagation

Various explants like nodal segments, shoot tips and cotyledonary nodes were used for the study.

3.7.2 Collection and preparation of explants

3.7.2.1 *In vitro* seedlings

Mature nuts (var. Priyanka, Poornima, Amrutha) of cashew were collected during the month of January-March from the Cashew Research Station, Madakkathara. After surface sterilization seeds were then germinated aseptically.

3.7.2.2 Mature trees

Nodal segments were collected from 7-8 years old cashew trees grown in the field under natural day light and day length conditions.

3.8 EXPLANTS FROM FIELD PLANTS

3.8.1 Standardization of surface sterilization

Nodal segments were taken from field grown cashew plants. The segments were washed thoroughly with tap water and kept in running tap water for 1 hr. The nodal segments were then treated with 1 per cent cetrimide for 15 min. and then with 1 per cent bavistin for 15 min. To standardize the surface sterilization of explants, $HgCl_2$ at varying concentrations (0.05 and 0.1 %) were tried at varying time intervals.

Different time intervals tried for the surface sterilization of nodal segments are as follows.

Treatment	Time interval (minutes)
T_1	2
T_2	3
T_3	5
T_4	7
T_5	10

Survival per cent was recorded after three weeks of culture.

3.8.2 Influence of basal media

Nodal segments were inoculated into MS and ½ MS media. Bud break per cent was recorded after four weeks of culture.

3.8.3 Influence of season on survival and bud break

Explants were collected from January to December 2007. Percentage of survival and bud break were recorded separately after 5-6 weeks of culture.

3.8.4 Culture establishment

Nodal segments taken from 7-8 year old field grown cashew plants were established in different media composition. The number of days for bud burst were recorded after 1-4 weeks after culture.

3.8.5 Multiplication and elongation

Nodal segments with buds were elongated in MS with different concentrations of growth regulators. Observations regarding the elongation obtained, number of multiple shoots/node and number of elongated shoots/node were recorded after 5-6 weeks of culture.

3.8.6 Rooting

Rooting was tried in MS media supplemented with different concentration of auxins are as follows.

Treatment	Media
T_1	½ MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA
T_2	½ MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IAA
T_3	½ MS + 1 mg l^{-1} NAA + 0.5 mg l^{-1} IAA
T_4	½ MS + 1 mg l^{-1} NAA + 0.5 mg l^{-1} IBA
T_5	½ MS + 1 mg l^{-1} NAA + 1 mg l^{-1} IAA
T_6	½ MS + 1 mg l^{-1} NAA + 1 mg l^{-1} IBA
T_7	½ MS + 0.5 mg l^{-1} IBA + 0.5 mg l^{-1} IAA
T_8	½ MS + 0.5 mg l^{-1} IBA + 1 mg l^{-1} IAA
T_9	½ MS + 1 mg l^{-1} IBA + 0.5 mg l^{-1} IAA
T_{10}	½ MS + 1 mg l^{-1} IBA + 1mg l^{-1} IAA
T_{11}	½ MS + 1.5 mg l^{-1} IBA + 1mg l^{-1} IAA
T_{12}	½ MS + 1.5 mg l^{-1} IBA + 1.5 mg l^{-1} IAA

3.9 Surface sterilization of nuts

Surface sterilization of nuts was done in order to make the nuts free of microorganisms. Nuts were washed thoroughly with tap water and then with water to which a few drops of Teepol were added. The nuts were then treated with two per cent Cetrimide for 45 min and then with 2.5 per cent Emisan for 1 hour.

In all treatments, nuts were submerged in sterilant for the required period and with frequent agitation. After the suface sterilization, solution was drained off, nuts were washed free of the chemical sterilant using distilled water. Then the nuts were transferred to sterile water, soaked in sterile water for 4 days. Surface sterilization was carried out under aseptic conditions in a laminar air flow cabinet. The soaked nuts were then treated with 0.1 per cent mercuric chloride for 30 min, dipped in alcohol and flamed.

The embryo with the cotyledons was excised carefully and inoculated into MS medium supplemented with 0.5 per cent charcoal, three per

cent sucrose and at varying levels of growth regulators. Observations were made on percentage of germination. The growth regulator combinations tried for establishment of nuts are as follows.

Treatment	Media
T_1	Full MS + 2.5 mgl^{-1} kinetin + 0.5 mgl^{-1} NAA
T_2	Full MS + 3.5 mgl^{-1} kinetin + 1 mgl^{-1} NAA

From the *in vitro* grown seedlings, cotyledonary nodes (~ 1.5 cm), nodal segments (~ 1.5 cm) and shoot tips (~ 3.0 cm) were cut and separated out. Cotyledonary nodes, nodal segments and shoot tips were taken from one month old seedlings.

From the full grown mature trees, nodal segments (~ 1.5 cm) and shoot tips (~ 1.5 cm) were cut and separated out from newly produced flushes.

3.10 EXPLANTS FROM *IN VITRO* SEEDLINGS

3.10.1 CULTURE ESTABLISHMENT AND MULTIPLICATION

Cotyledonary nodes, nodal segments and shoot tips taken from one month old seedlings were established in MS with different combinations of auxins and cytokinins.

The growth regulator combinations tried for culture establishment and multiplication are as follows:

Treatment	Media
T_1	Full MS + 1 mgl^{-1} BA + 0.5 mgl^{-1} IAA
T_2	1.5 mgl^{-1} BA + 1 mgl^{-1} IAA
T_3	2 mgl^{-1} BA + 1 mgl^{-1} IAA
T_4	2.5 mgl^{-1} BA + 1 mgl^{-1} IAA
T_5	3 mgl^{-1} BA + 1.5 mgl^{-1} IAA

The number of multiple shoot buds and the number of elongated shoots obtained were recorded separately after three weeks of culture.

3.10.2 ELONGATION

The clumps of shoot buds smaller than 1.5 cm were separated out and were elongated in MS with different combinations of cytokinins. The growth regulator combinations tried for elongation are as follows:

Treatment	Media
T_1	MS+2 mgl^{-1} BA + 400 mgl^{-1} glutamic acid
T_2	MS+2.5 mgl^{-1} BA + 400 mgl^{-1} glutamic acid
T_3	MS+3 mgl^{-1} BA + 400 mgl^{-1} glutamic acid

Observations regarding the elongation obtained and number of nodes were recorded after three weeks.

3.10.3 ROOTING

Rooting was tried in MS media supplemented with various concentrations of auxins. Pulsing was carried out before the inoculation of elongated shoots into media.

Pulsing of the elongated shoots was tried in the following combinations.

Treatment	Media	Time interval
T_1	½ MS + 12 mgl^{-1} IBA	30 s, 1 min, 1 hr, 8 hr, 16 hr, 24 hr, 36 hr, 40 hr, 72 hr
T_2	½ MS + 16 mgl^{-1} IBA	,,
T_3	½ MS + 20 mgl^{-1} IBA	,,
T_4	½ MS + 24 mgl^{-1} IBA	,,
T_5	½ MS + 10 mgl^{-1} NAA	,,
T_6	½ MS + 15 mgl^{-1} NAA	,,
T_7	½ MS + 20 mgl^{-1} NAA	,,

Rooting of the elongated shoots were tried in the following combinations

Treatment	Media
T_1	¼ MS + 200 mgl^{-1} glutamic acid + 1 mgl^{-1} IBA
T_2	¼ MS + 200 mgl^{-1} glutamic acid + 1.5 mgl^{-1} IBA
T_3	¼ MS + 200 mgl^{-1} glutamic acid + 2 mgl^{-1} IBA
T_4	¼ MS + 200 mgl^{-1} glutamic acid + 2.5 mgl^{-1} IBA
T_5	¼ MS + 200 mgl^{-1} glutamic acid + 1 mgl^{-1} IBA + 1 mgl^{-1} IAA
T_6	½ MS + 0.5 mgl^{-1} IBA + 0.5 mgl^{-1} IAA
T_7	½ MS + 1 mgl^{-1} IBA + 0.5 mgl^{-1} IAA
T_8	½ MS + 1 mgl^{-1} IBA + 1 mgl^{-1} IAA
T_9	½ MS + 0.5 mgl^{-1} IBA + 1 mgl^{-1} IAA

3.10.3.1 EFFECT OF *GLOMUS FASCICULATUM* ON *IN VITRO* ROOTING

Spores of *Glomus fasciculatum* were isolated from soil by wet sieving and decanting method. Spores were surface sterilized in chloramine T (2 per cent) solution for 10 minutes followed by streptomycin (0.02 per cent) for 10 minutes (Budi *et al*., 1999) and washed with sterile water. Surface sterilization of spores were carried out under aseptic conditions in a laminar air flow cabinet. Two types of explants were used (1) before the initiation of rooting (2) after the initiation of rooting. Thirty spores were inoculated per one explant and kept under 16 hrs photoperiod.

Observation regarding the per cent root colonization was recorded 30 days after inoculation of spores.

3.10.3.2 EFFECT OF *BACILLUS SUBTILIS* ON *IN VITRO* ROOTING

Twenty four hrs old *Bacillus subtilis* culture was used as inoculum. Bacterial inoculum (10^8 cfu ml^{-1}) was added into the media at two stages (1) after the initiation of roots (2) three days before the transplanting. Observation regarding elongation obtained was recorded after two weeks after inoculation.

3.10.3.3 ROOT INDUCTION WITH *AGROBACTERIUM RHIZOGENES*

Two strains of *Agrobacterium rhizogenes*, MTCC 532 and MTCC 2364 of agropine family were used for the present study.

Table 4. Details of strains obtained from IMTECH

MTCC No.	2364	532
Type	B	B
Genus Name	*Agrobacterium*	*Agrobacterium*
Species including subspecies/ Variety	*rhizogenes*	*rhizogenes*
Strain Designation	30200	15834
Source of Isolation	Stem gall	Not mentioned
Culture Received From	DSM	ATCC
Equivalent Number of other culture collections	DSM 30200	ATCC 15834
Growth Medium	*Xanthomonas* medium	Nutrient Agar
Growth condition	Aerobic	Aerobic
Growth temperature	25°C	25°C
Special features/applications	Not mentioned	Virulent

3.10.3.3.1 Culturing of *A. rhizogenes* strains

The bacterial strains were cultured on four different media, Luria Bertani Agar (LBA), Yeast Extract Mannitol (YEM), Yeast Extract Broth (YEB) and Nutrient Agar (NA) to select a suitable growth medium. The solid media was melted, cooled to 40-50°C and poured into sterilized petriplates. Each strain was streaked on plates containing the respective media. The growth rate of bacteria on each medium was observed. To study the influence of temperature on the growth of *A. rhizogenes* strains, one set of the culture was incubated at room temperature (30°C) and another set in the culture room at 26 ± 2°C. The growth of bacteria in different culture conditions was observed.

3.10.3.3.2 Isolation of single cell colonies

The bacterial strains were streaked on appropriate culture medium so as to isolate single cell colonies. To streak the bacteria on to the plate, the transfer loop was flamed and cooled repeatedly three times. The loop was then plunged into a well-grown bacterial colony. The lid of the petriplate containing sterilized, solid growth medium was lifted from one side and the loop loaded with bacteria was drawn gently on about one-third of the plate surface, to bring three lines close together, but separated from each other. The loop was again flamed, cooled and drawn across one end of the second streaked area and the remaining one-third area of the plate was streaked. The third sector was also streaked similarly. The plate was closed, sealed with parafilm and kept in the culture room on a rack. Observations regarding growth of bacteria were documented.

3.10.3.3.3 Screening of *A. rhizogenes* strains for antibiotic sensitivity

The *A. rhizogenes* strains used for the study were tested for resistance to antibiotics. The antibiotics used for testing resistance were cefotaxime, ampicillin and carbenicillin. Nutrient agar medium was selected for the study. The sterilized media was melted and cooled to 40-50°C and supplemented with 50, 100, 200, 300, 400 and 500 mgl^{-1} of each antibiotic separately. The medium was poured into petriplates and allowed to solidify. The petriplates containing solidified medium was divided by marker lines into three sectors. The bacteria from a single cell colony were streaked on the respective parts in each petriplate. Bacterial strains were also streaked in NA medium without any antibiotics, to be used as control.

3.10.3.3.4 Maintenance of strains

The strains were maintained as stabs and glycerol stocks. The best growth medium (YEB or NA) containing antibiotic was used.

3.10.3.3.5 Evaluation of the sensitivity of explants to various antibiotics

Different explants like shoot tips and nodal segments were tested for their sensitivity to various concentrations of antibiotics. The antibiotics used were cefotaxime and ampicillin. The wounded explants were cultured in solid MS medium containing 100, 200, 300, 400, 500, 600 and 700 mg l^{-1} concentrations of either ampicillin or cefotaxime. As control the explants were cultured in MS solid medium without antibiotics. Observations regarding the growth and the response of explants were recorded.

3.10.3.3.6 Standardization of inoculation method

Two types of bacterial inoculum were used for the study. In one method of inoculation, the bacterium from isolated single cell colonies were used as the bacterial inoculum (hereinafter referred to as Direct Inoculation Method or DIM). The bacterial suspension was used as the inoculum in the other method (hereinafter referred to as the suspension culture inoculation method or SM).

3.10.3.3.6.1 Direct Inoculation Method

In this method, bacterium from isolated single cell colonies was used as the inoculum. Wounds were made on the explant using a sterile blade. Cuts were made on basal part of the nodal segments and shoot tips using sterile blade loaded with inoculum. The explants were then blotted with a sterile blotting paper and placed on solid MS medium without growth regulators contained in the

petri plates. As control, explants were wounded with a sterile blade, blotted dry and placed on growth regulator free MS medium.

3.10.3.3.6.2 Suspension culture inoculation method

The pre-cultured explants were wounded first using sterile blade and injection needle. The *Agrobacterium* suspension were prepared (OD_{600} ~ 1.0) in a jam bottle. The wounded explants were immersed in the suspension for 8 hr. Black cloth was used to cover the jam bottle containing explants with *Agrobacterium* suspension. The explants were then blotted dry using sterile blotting paper and placed on solid MS medium without growth regulators taken in the test tubes. The control explants were wounded and dipped in liquid MS medium for 8 hr and then cultured on solid MS contained in the petriplates.

3.10.3.3.7 Co-cultivation of explants with *Agrobacterium*

The infected explants were blotted dry using sterile blotting paper and were placed on growth regulator free MS solid medium in test tubes. The infected explants were then co-cultured in dark at 26 ± 2°C for 1-3 days in the culture room. Darkness was provided in the culture rack using black cloth. Observations regarding the growth of bacterium on media were noted.

3.10.3.3.8 Influence of co-culture period

The explants infected by DIM and SM were co-cultured for 1-3 days at 26 ± 2.0°C under dark photoperiod for efficient transformation. Transformation percentage obtained and the responses of different explants with respect to different co-culture period were recorded.

3.10.3.3.9 Influence of acetosyringone

Three methods were used to study the influence of acetosyringone on hairy root induction. The infected explants were co-cultured in acetosyringone containing media. Acetosyringone (3', 5'-dimethoxy'4'-hydroxyacetophenone) dissolved in dimethyl sulfoxide (DMSO) was used as the stock. Autoclaved MS solid medium prepared in the conical flask was melted and cooled to 40-50°C. From the stock, acetosyringone at the rate of 10 and 20 mM was added to the medium aseptically, shaken well and poured to sterile test tubes and solidified.

Explants were infected by DIM or SM and then co-cultured in dark for two days in solid MS media containing 10 and 20 mM acetosyringone (hereinafter referred as Direct Inoculation and Co-cultivation with Acetosyringone or DICA method and Suspension culture and Co-cultivation with Acetosyringone or SCA method respectively). As control, one set of explants inoculated by DIM and SM were placed in co-culturing media without acetosyringone under dark photoperiod for two days.

In the third method, *Agrobacterium* suspension was prepared. When OD_{600} of the bacterial suspension reached approximately one, the suspension was collected in an Oak ridge centrifuge tube and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded and the bacterial pellet was washed thrice with liquid MS. The pellet obtained was resuspended in 5.0 ml growth regulator free liquid MS medium. To the suspension 10 and 20 mM acetosyringone was added and kept as such for two hours for activating the bacteria. After that, the suspension was made up to 30 ml and transferred to petriplates. Wounded explants were immersed in the suspension for 8 hr. The explants were blotted dry using sterile blotting paper and co-cultured in dark for three days of solid MS medium containing 10 and 20 mM acetosyringone.

The number of roots per explant and the rooting percentage were recorded 25 days after infection.

3.10.3.3.10 Culture media and conditions for root induction

The explants after bacterial co-culture were washed three times successively with MS liquid medium containing 500 mgl^{-1} cefotaxime. To the sterilized MS liquid medium prepared in the jam bottles, 500 mgl^{-1} cefotaxime was added aseptically. The co-cultured explants were washed by immersing in the media containing antibiotics with intermittent shaking. After five minutes, the explants were transferred to fresh MS media containing the antibiotic and the process of washing was repeated two more times.

After washing, the explants were blotted dry using sterile blotting paper. The explants were then transferred to solid MS medium containing the bacteriostatic agent for the complete elimination of bacteria (MS + 500 mgl^{-1} cefotaxime). The explants were further cultured at 26 ± 2°C under diffused light.

If the bacterial growth was seen after a few days, the explants were again washed with liquid MS medium containing the antibiotic. This was repeated until no bacterial growth was seen on the media. The control explants were also treated similarly.

3.10.3.3.11 Standardization of explants for efficient root induction with *A. rhizogenes*

Different explants like shoot tips and nodal segments were infected by all the two strains of *Agrobacterium rhizogenes* (MTCC 532 and MTCC 2364) available. The infection was carried out using different inoculation methods and co-cultured for different durations (1-3 days) under diffused light. Rooting

percentage obtained in the case of each explants was recorded. The mean number of roots per explant under each condition was recorded after 30 days.

Observations regarding number of days for root induction, percentage of rooting, number of roots per explant recorded after four weeks.

3.10.4 HARDENING AND PLANTING OUT

The *in vitro* rooted plantlets obtained, were taken out of the test tubes using forceps, after soaking the media in the test tubes in water for five min. The solidified media from the plantlets was washed out under running tap water. The plantlets were then planted in small earthern pots filled with sterilized sand. To enhance the transplantation success, microorganisms like *Glomus fasciculatum, Bacillus subtilis* and their combinations were tried at the time of planting out. Hardening and planting out were tried in following combinations. They were transferred to the green house.

Treatments		Time of inoculation
T_1	Control	At the time of planting out
T_2	*Glomus fasciculatum*	"
T_3	*Bacillus subtilis*	"
T_4	*Glomus fasciculatum* + *Bacillus subtilis*	"

3.10.4.1 MASS MULTIPLICATION OF AM FUNGAL INOCULUM

Pure culture of VAM was mass multiplied in sterile soil and sand (2:1). The maize seeds were surface sterilized with sodium hypochlorite (0.1 per cent) for 10 minutes and washed with sterile water. The sterilized soil and sand (2:1) was added to earthern pots (capacity 3 Kg). Top surface of soil of 1cm

thickness was removed and vermiculite culture based VAM was applied. Surface sterilized maize seeds were sown and covered with soil. Plants were watered daily using sterile distilled H_2O and maintained for 90 days. The shoot portions of the maize plants were cut and removed after 90 days after sowing. The roots were cut into small pieces and mixed thoroughly with the soil. The AMF per cent root colonization was assessed to detect the infection of *Glomus fasciculatum* in maize roots. The infected root bits, hyphae, and rhizosphere soil from the pots were used as inoculum for further studies.

3.10.4.2 PER CENT ROOT COLONIZATION OF CASHEW

The per cent root colonization of AMF was assessed using the method described by Phillips and Hayman (1970). The roots were washed in tap water to remove the adhering soil particles and were then cut into bits of one cm length and fixed in Formalin: Acetic acid: Alcohol mixture (FAA)(Annexure VII). The root bits fixed in FAA were washed thoroughly in water to remove the fixative. The washed root bits were softened by simmering in 10 per cent KOH at 90°C for 1 hr. After cooling, the excess KOH was washed off in tap water and then neutralized with 2 per cent HCl. The root bits were then stained with 0.05 per cent trypan blue in lactophenol (Annexure VIII) for three minutes. The excess stain from the root tissue was removed by cleaning in lactophenol. The root bits were examined under microscope (40X) for AMF colonization. The per cent AMF colonization was determined using the following formula.

$$\text{Per cent root colonization} = \frac{\text{Number of infected root segments}}{\text{Total number of root segments observed}} \times 100$$

3.10.4.3 ISOLATION OF AM FUNGAL SPORES

The AM fungal spores were isolated from the rhizosphere soil by wet sieving and decanting method (Gerdemann and Nicolson, 1963). About 250g

of rhizosphere soil was suspended in 1000 ml water and stirred well. After settling of the heavier particles, the supernatent was filtered through a set of sieves of size 425, 250, 105 and 45 microns (Jayant test sieves, Jayant Scientific Ind. Mumbai, 400 002). Finally the soil suspension present in 45 and 105 micron sieves were transferred to 100 ml beakers separated by gentle washing. The spore suspension was filtered through Whatmann No.1 filter paper. The filter paper containing spores was placed in a petri dish and observed under stereo microscope. The number of similar spores were picked and counted separately based on the shape and colour of spores. The isolated spores were transferred to moistened filter paper for further studies.

3.10.4.4 DETECTION OF AMF IN CASHEW ROOTS

Glomus fasciculatum present in the roots of the transplanted plantlets were detected by amplification with PCR. Template DNA for PCR was obtained from crushed root samples (8 to 20 mg) or inoculum propagules (10 to 20 mg) by boiling each sample in 800 µl of extraction buffer (1 M Tris-HCl, pH 8.5) for 15 min. DNA template from roots was also obtained by adding Chelex 100 resin, to a final concentration of 5 per cent, to the extraction buffer prior to boiling. The resin was removed by centrifugation at 12,000 rpm for 20 s before the DNA template was used for PCR. The processed samples were either immediately used for PCR or frozen at -20^0C.

3.10.4.4.1 Detection of AMF in cashew roots with PCR

After isolation of good quality of genomic DNA from *Glomus fasciculatum*, PCR was performed for the amplification of 18S rDNA. Amplification of the selected region from a complex DNA mixture is carried out *in vitro* by the DNA polymerase I from *Thermus aquaticus*, a bacterium that lives in hot springs. 18S rDNA gene of *Glomus fasciculatum* was amplified using two primers: VANS1 (5'-GTCTAGTATAATCGTTATACAGG-3') and NS21 (5'-

AATATACGCTATTGGAGCTGG-3'). PCR products were separated on 1 per cent agarose gel. The following PCR template was prepared.

1. Diluted 1µl of DNA (≈25 ng) to 10 µl with sterile H_2O.
2. One microlitre of diluted DNA was added in a new PCR tube.
3. A Master Mix was prepared and the reagents were added in the sequential order as shown in Table 5.
4. The contents were mixed well and briefly centrifuged.
5. Twenty four micro litres of the Master Mix was aliquotted into 0.2 ml microfuge tube.

The following PCR programme was run immediately.

Step 1. Initial denaturation at 95°C for 3 minutes.

Step 2. Denaturation at 94°C for 60 seconds
Step 3. Primer Annealing at 50°C for 45 seconds
Step 4. Primer extension at 72°C for 60 seconds
} 35 cycles

Step 5. Final extension at 72°C for 10 minutes

Table 5. Preparation of PCR master mix

Components	**Vol. per reaction (µl)**
Genomic DNA (25 ng)	1.0
10 X *Taq* assay buffer containing $MgCl_2$	2.5
d NTP mix (10 mM each)	1.0
VANS1 (Forward primer)	1.0
NS21 (Reverse primer)	1.0
Taq DNA polymerase (0.5u)	2.0
Autoclaved distilled water	16.5
Total volume	**25.0**

A momentary spinning was given for the reaction and set in thermal cycler for polymerase chain amplification under suitable programme with heated lid condition. The PCR product was checked on 1 per cent agarose gel and documented.

Results

4. RESULTS

The results of the study on "Management of recalcitrancy in *in vitro* cultures of cashew (*Anacardium occidentale* L.) are presented in this chapter.

4.1 ISOLATION, IDENTIFICATION AND MANAGEMENT OF ENDOPHYTIC AND COVERT BACTERIA

4.1.1 ISOLATION OF ENDOPHYTIC BACTERIA FROM *IN VITRO* CULTURES

The protocol suggested by Dinakaran *et al.* 2003 was used for the isolation of endophytic bacteria from *in vitro* cultures of cashew. Serial dilution and plating of ground sample in phosphate buffer revealed presence of one bacterium (Plate 1,2). It was detected in all the *in vitro* cultures (10 cultures). Hereafter it was named as KAU-EC1.

4.1.2 ISOLATION OF COVERT BACTERIA FROM *IN VITRO* CULTURES

Among the 20 *in vitro* cultures of cashew, four turned index positive for medium during the first screening (after growing the cultures in antibiotic medium for one month) (Plate 3). Dilution plating revealed two bacteria in each of the cultures (Plate 4, 5). These bacteria were purified by subsequent streaking. Hereafter these bacteria were named as KAU-CC1 and KAU-CC2. Single colonies of pure cultures were stab inoculated in NA media in cryostorage vials and maintained under refrigerated conditions.

Plate 1. Contamination with Endophytic bacteria

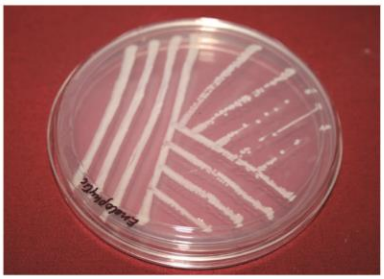


Plate 2. Endophytic bacteria - KAU - EC1

Plate 3. Isolation of covert bacteria from *invitro* cultures

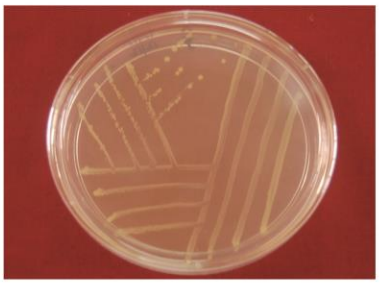


Plate 4. Covert bacteria - KAU-CC1

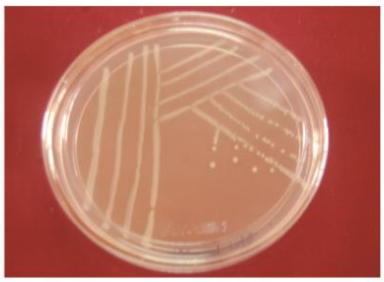


Plate 5. Covert bacteria - KAU-CC2

4.1.3 MORPHOLOGICAL CHARACTERIZATION

4.1.3.1 Growth in liquid medium

All the isolates showed finely dispersed growth through out nutrient agar broth and there was uniform turbidity showing aerobic nature of the bacterium.

4.1.3.2 Gram staining

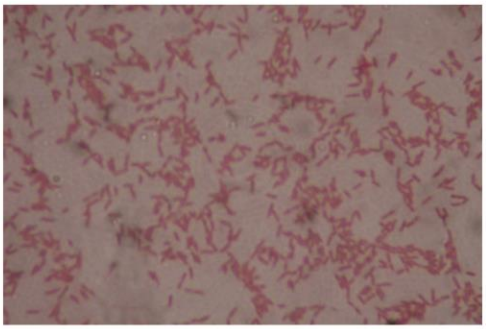
Cells were rod-shaped, occurred singly and appeared red. Gram staining reaction indicated Gram negative nature (Plate 6). This was further confirmed by solubility in 3 per cent KOH. The cultures became viscous and thin strands could be looped out in the presence of KOH.

4.1.4 INTRINSIC ANTIBIOTIC RESISTANCE

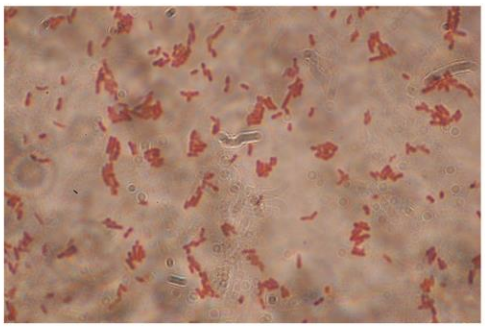
KAU-EC-1 was resistant to streptomycin, cefotaxime, gentamycin, ampicillin, nalidixic acid and tetracyclin at the highest concentration tested. KAU-CC-2 was also resistant to streptomycin, cefotaxime, gentamycin, ampicillin, nalidixic acid and tetracyclin at the highest concentration tested and KAU-CC-1 was sensitive to streptomycin, cefotaxime and gentamycin (Plate 7). Response of KAU-CC1, KAU-CC2, and KAU-EC1 are shown in the Table 6. These antibiotics were also added to the culture medium at various stages. Based on the data, the antibiotic resistance markers were identified for each bacteria given in Table 7.

4.1.5 MOLECULAR IDENTIFICATION

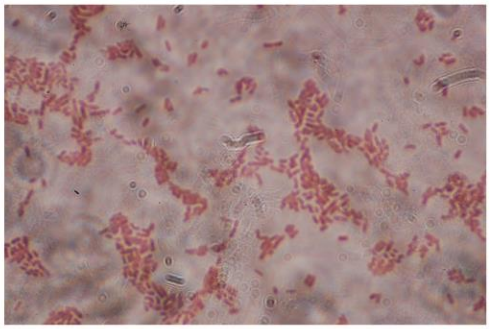
Bacterial genomic DNA of covert and endophytic bacteria were isolated, purified and subjected to PCR for 16S rDNA amplification.



KAU - EC1

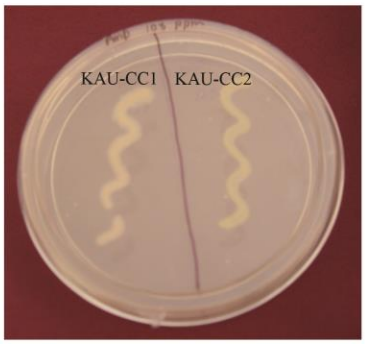


KAU - CC1

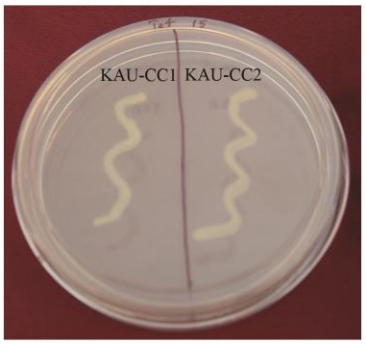


KAU - CC2

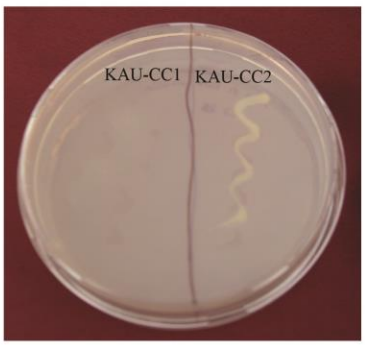
Plate 6. Gram staining of KAU-EC1, KAU-CC1 and KAU-CC2



Ampicillin 100 ppm



Tetracyclin 15 ppm



Cefotaxime 250 ppm

Nalidixic acid 15 ppm

Plate 7. Evaluation of sensitivity of KAU-CC1 and KAU-CC2 to various antibiotics

Table 6. Intrinsic antibiotic resistance patterns of the isolates

Sl. No	Antibiotics	Concentration (mg ml^{-1})	KAU-EC-1	KAU-CC-1	KAU-CC-2
1	Ampicillin	25	+	+	+
		50	+	+	+
		100	+	+	+
2	Cefotaxime	50	+	-	+
		100	+	-	+
		250	+	-	+
3	Gentamycin	5	+	-	+
		10	+	-	+
		25	+	-	+
4	Nalidixic acid	5	+	+	+
		10	+	+	+
		15	+	+	+
5	Streptomycin	50	-	+	+
		100	-	+	+
		250	-	+	+
6	Tetracyclin	10	+	+	+
		15	+	+	+
		25	+	+	+

+ growth present (resistant) - growth absent(susceptible)

Table 7. Antibiotic sensitivity markers developed for isolates

Isolates	Antibiotic resistance
KAU-CC-1	Amp^{R}_{100} Cef $^{R}_{250}$ Gen^{R}_{5} Nal^{R}_{15} Str^{S}_{50} Tet^{R}_{25}
KAU-CC-2	Amp^{R}_{100} Cef $^{R}_{250}$ Gen^{R}_{25} Nal^{R}_{15} Str^{R}_{250} Tet^{R}_{25}
KAU-EC-I	Amp^{R}_{100} Cef $^{R}_{250}$ Gen^{R}_{25} Nal^{R}_{15} Str^{R}_{250} Tet^{R}_{25}

Amp-Ampicillin, Cef-Cefotaxime, Gen-Gentamycin, Nal-Nalidixic acid, Str-Streptomycin, Tet-Tetramycin

Table 8. Quality and quantity of DNA isolated from endophytic and covert bacteria

Sample	Absorbance at 260 nm	Absorbance at 280 nm	OD_{260}/OD_{280}	Quantity (ng/µl)
KAU-EC1	4.83	2.72	1.77	241.3
KAU-CC1	1.39	0.8520	1.64	69.7
KAU-CC2	1.08	0.585	1.85	54.1

KAU-EC1 : KAU - endophytic cashew1

KAU-CC1 : KAU- cashew covert1

KAU-CC2 : KAU- cashew covert 2

4.1.5.1 Isolation and purification of genomic DNA

The procedure reported by Schleif and Wensink (1981) was used for isolation of bacterial genomic DNA. The quality of DNA isolated was tested using agarose gel electrophoresis. Better quality of DNA was indicated by discrete bands (Plate 8, 9).

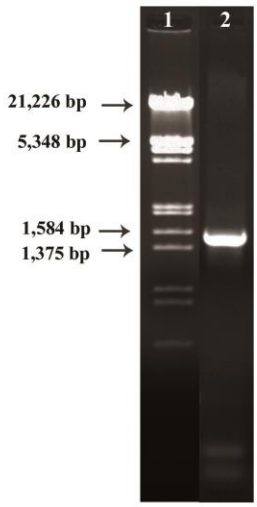
The quality of DNA of endophytic and covert bacteria was estimated spectrophotometrically (Table 8), KAU-CC2 gave a ratio greater than 1.8 at OD_{260}/OD_{280}, indicating good quality, pure DNA. KAU-EC1 and KAU-CCI gave a ratio 1.77, 1.64 respectively.

4.1.5.2 PCR

16S rDNA of different isolates were amplified by using universal primer 16S $_{43-63}$, 16S $_{1404-1387}$. Analysis of PCR amplification was carried out on 1.2 per cent agarose gel. Size of the amplified product was 1.3 kb (Plate 10, 11).

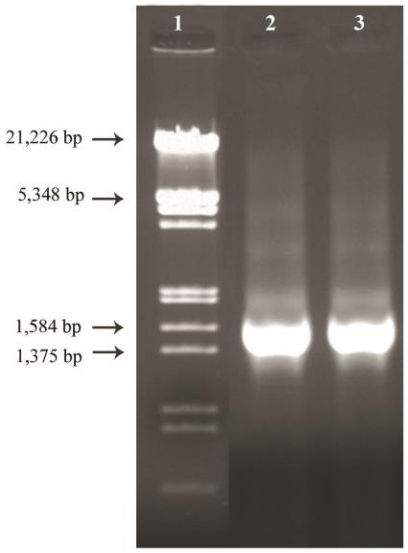
4.1.5.3 Gel elution

Distinct bands obtained in PCR amplification were eluted and checked on 1.2 per cent agarose gel. Distinct band with good concentration was observed (Plate 12, 13) similar to that obtained during PCR amplification, thus indicating good recovery of fragments from the gel and its suitability for cloning in a vector. The quantity estimated was 4 ng, 3 ng, 5 ng of DNA μl^{-1} based on absorbance at 260 nm and 280 nm.



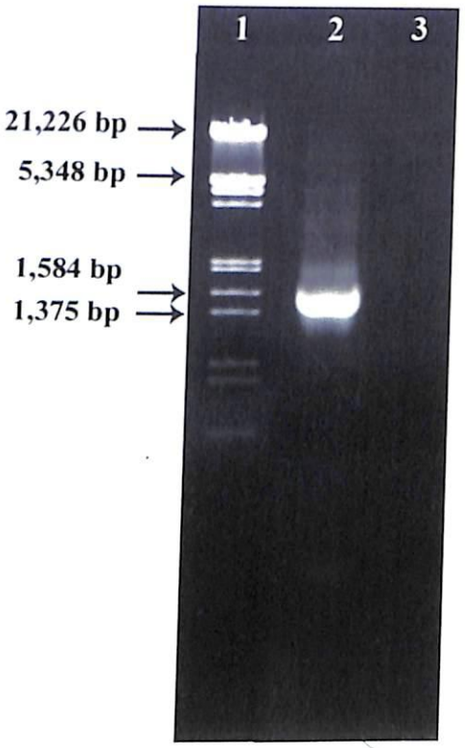
Lane 1 - λ DNA / *Eco* RI *Hae* III digest
Lane 2 - DNA of KAU-EC1

Plate 8. DNA of KAU-EC1 isolated by Schleif and Wensink method



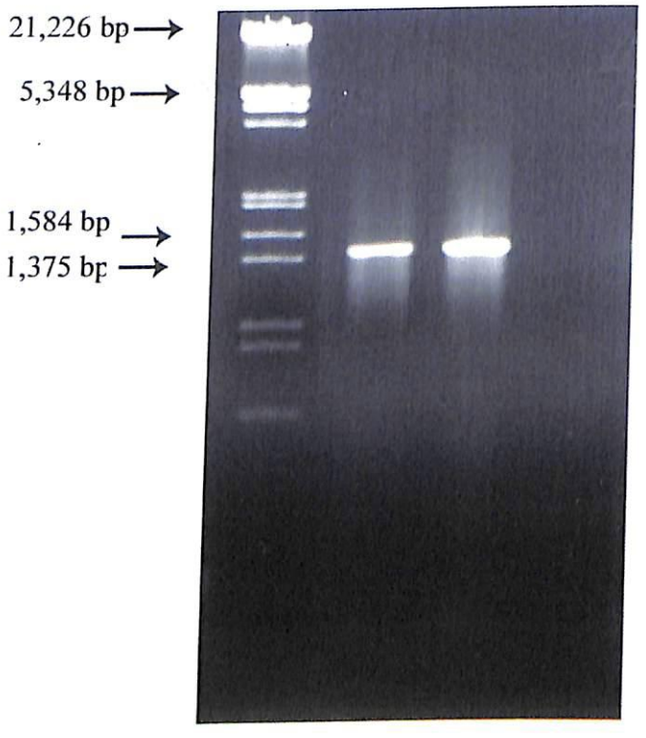
Lane 1 - λ DNA / *Eco* RI *Hae* III digest
Lane 2 - DNA of KAU-CC1
Lane 3 - DNA of KAU-CC2

Plate 9. DNA of KAU-CC1 and KAU-CC2 isolated by Schleif and Wensink method



Lane 1 - λ DNA / *Eco* RI *Hae* III digest
Lane 2 - PCR product of KAU-EC1
Lane 3 - Blank

Plate 10. Amplification of 16S rDNA of KAU-EC1 with $16S_{43-63}$ and $16S_{1404-1387}$



Lane 1 - λ DNA / *Eco* RI *Hae* III digest
Lane 2 - PCR product of KAU-CC1
Lane 3 - PCR product of KAU-CC2
Lane 4 - Blank

Plate 11 Amplification of 16S rDNA of KAU-CC1 and KAU-CC2 with 16S43-63 and 16S1404-1387

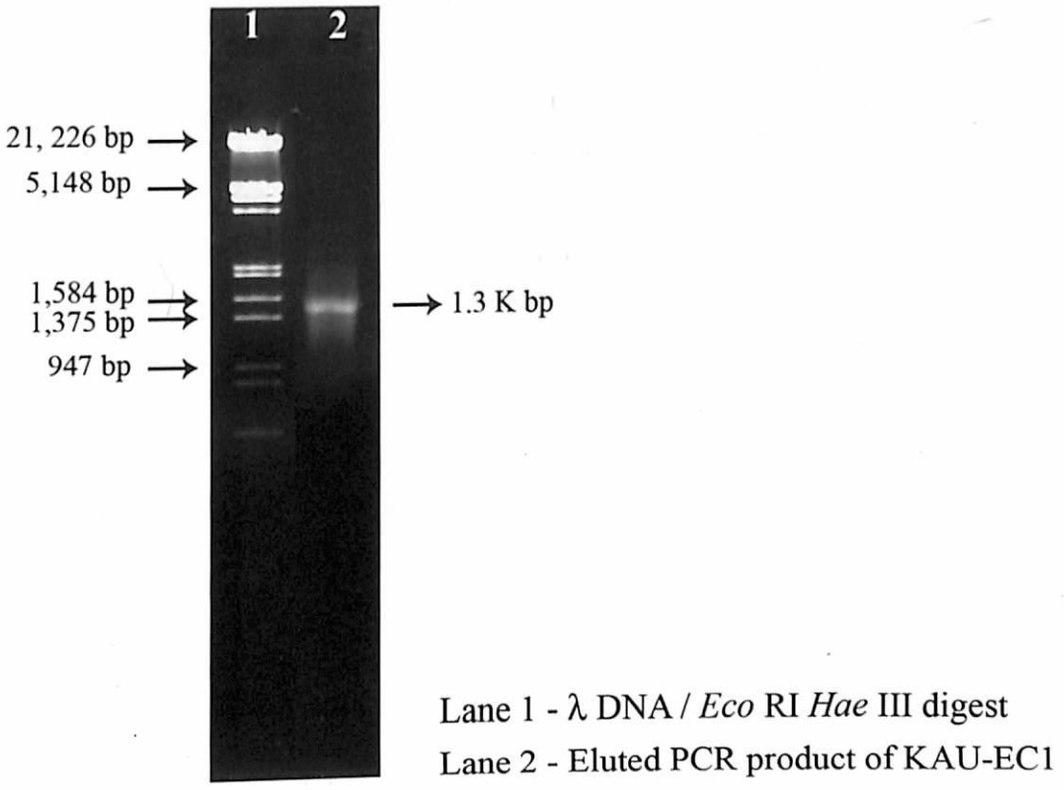


Plate 12. Eluted PCR product obtained from KAU-EC1

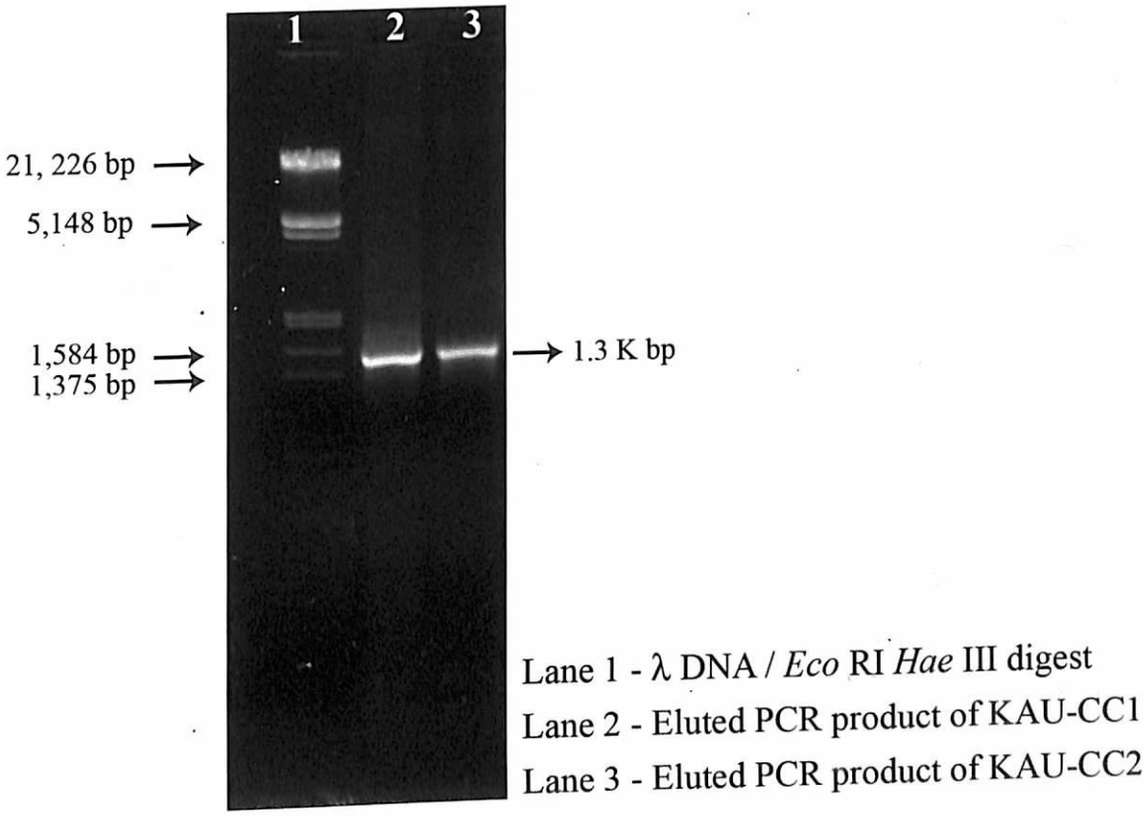


Plate 13. Eluted PCR product obtained from KAU-CC1 and KAU-CC2

4.1.5.4 Transformation

4.1.5.4.1 Preparation and screening of competent cells

The competent cells prepared in section 3.2.10.1 were checked for competence by transferring the plasmid (pUC 18) having ampicillin resistance. A large number of blue colonies were obtained (Plate 14 A) which indicated a high degree of transformation efficiency. Thus the competent cells prepared were found ideal for cloning amplicons.

4.1.5.4.2 Transformation of ligated product

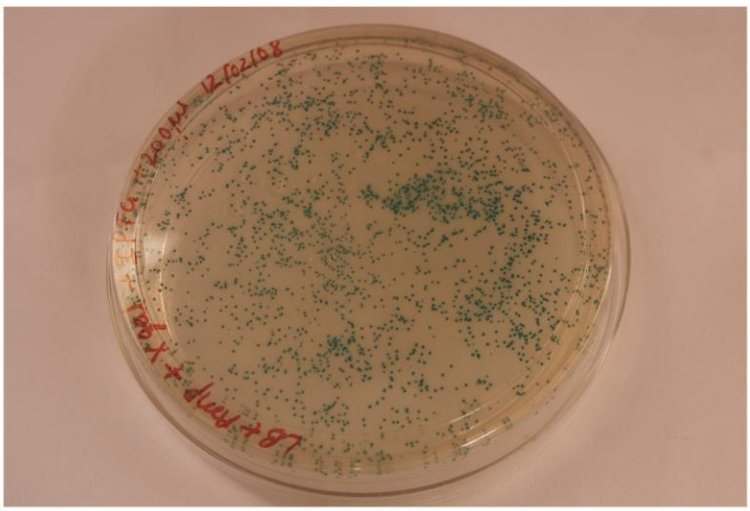
The ligated product was used to transform the prepared competent cells using the heat shock method and was incubated at 37^{o}C.

Blue and white colonies were determined after overnight incubation when the transformed cells were cultured in LB/ ampicillin media, the two plates overlaid with x gal IPTG (Plate 14 B).

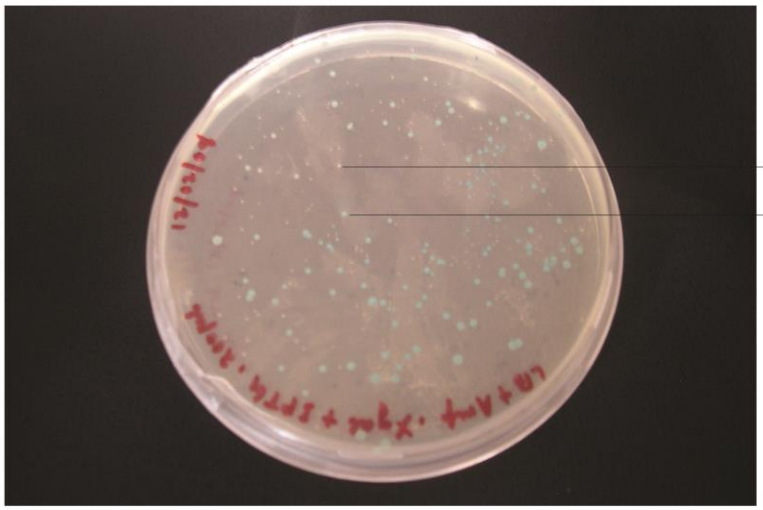
Table 9. The details of recombinants obtained in cloning of three bacteria

Details	KAU-EC1	KAU-CC1	KAU-CC2
No. of white colonies	66	59	72
No. of blue colonies	91	88	98
Total number of colonies	157	147	170
Recombination efficiency	42	40	42

Efficiency of recombinant of KAU-EC1, KAU-CC1 and KAU-CC2 are 42, 40 and 42 respectively. The white colonies were transferred to a new LB/ampicillin plate.



A. Checking the competence of *E.coli* cells



B. Blue white screening of transformed *E.coli* cells

White colonies are transformed and blue colonies are non-recombinant

Plate 14. Efficiency of the competent cells and screening of transformants

4.1.5.5 Screening of the transformed colonies

The plates containing the transformed colonies were screened for recombinant plasmid. The plasmid DNA isolated from white and blue colonies when viewed after electrophoresis in 0.8 per cent agarose gel indicated difference in molecular weight. Size of the recombinant plasmid was 1.3 kb and that of non recombinant plasmid was less than 1.3 kb. This confirmed the presence of the insert in the plasmid.

4.1.5.6 Confirmation of recombination by PCR

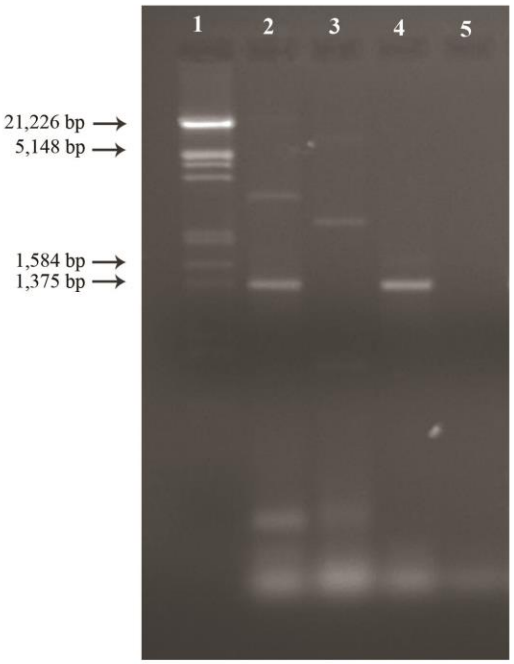
The plasmid was further checked for the presence of insert by PCR amplification using M13 primers. The PCR products when checked on 1.2 per cent agarose gel showed amplicons of different sizes. Higher molecular weight bands were obtained in PCR products of plasmid DNA isolated from white colonies than the PCR products of those isolated from blue colonies (Plate 15, 16). This confirmed the presence of the insert in the plasmid.

4.1.5.7 Sequencing

Recombinant plasmids of KAU-EC1, KAU-CC1 and KAU-CC2 were sent for automated sequencing. The sequencing facility available at Bangalore Genei was utilized. The sequence data were obtained from the firm within 15 days in the form of nucleotide sequence.

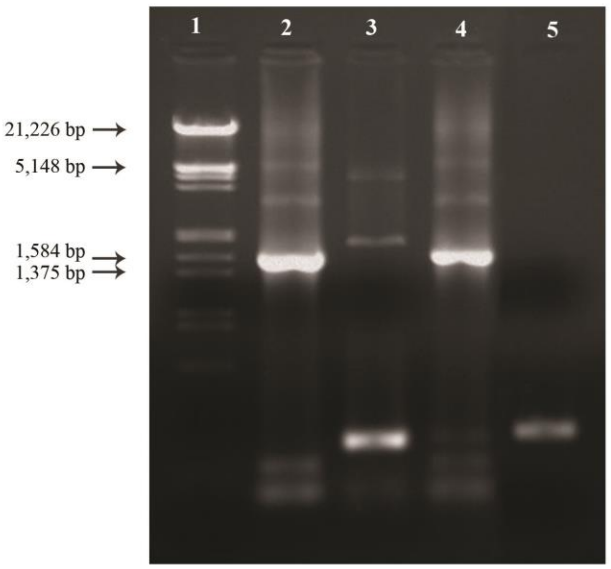
4.1.5.8 Sequence data analysis

The sequence data obtained from the sequencing agency for KAU-EC1, KAU-CC1 and KAU-CC2 are given in Annexure IX and their interpretations are given hereunder



Lane 1 - λ DNA / *Eco* RI *Hae* III digest
Lane 2 - White plasmid of KAU-EC1
Lane 3 - Blue plasmid of KAU-EC1
Lane 4 - DNA of KAU-EC1
Lane 5 - Blank

Plate 15. Confirmation of recombination in plasmid DNA of KAU-EC1 with M 13



Lane 1 - λ DNA / *Eco* RI *Hae* III digest
Lane 2 - White Plasmid DNA of KAU-CC1
Lane 3 - Blue plasmid
Lane 4 - White plasmid DNA of KAU-CC2
Lane 5 - Blank

Plate 16. Confirmation of recombination in plasmid DNA of KAU-CC1 and KAU-CC2 with M 13

KAU-EC1

The sequence data obtained for the KAU-EC1 was of 646 bp in size and vector screening indicated vector sequence from 10 to 47 bp. The details of the results obtained are presented in Table 10 and Fig. 2. After vector and adaptor screening, the total sequence obtained for further analysis was 609 bp as detailed below.

Vector/Adapter eluted sequence

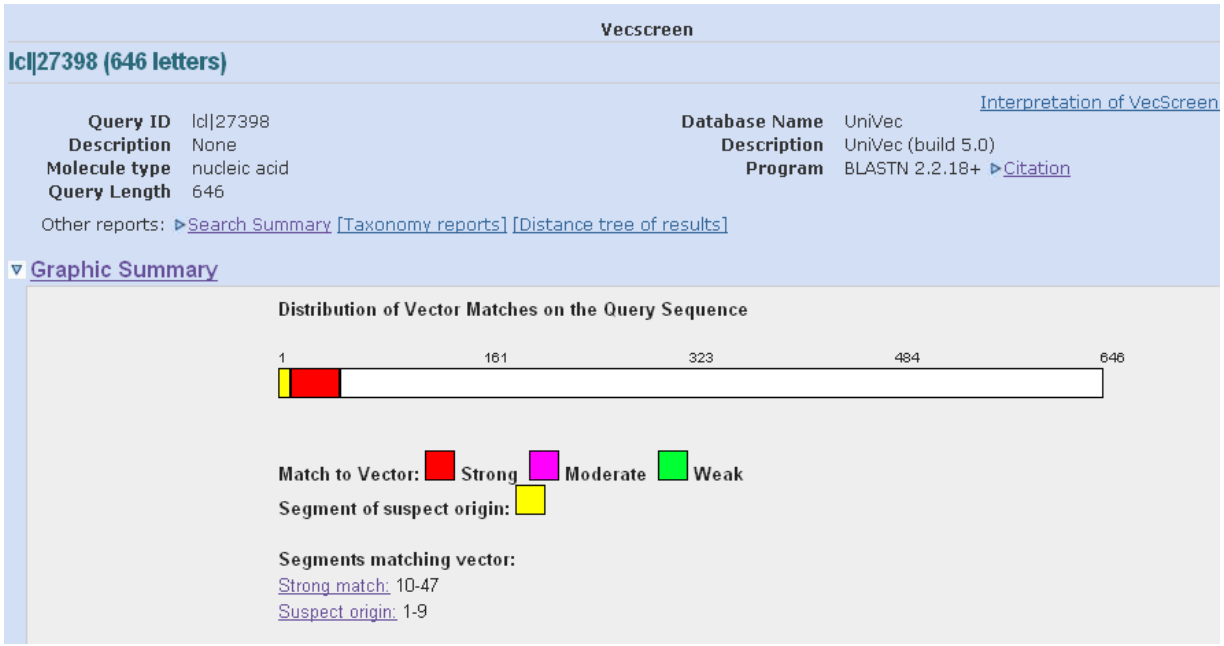
ACGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATCTGCCAGT
TTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACC
GCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTA
CCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCG
CCAAGGTTATTAACCTTAACGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGA
AGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAAT
ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGG
CTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCATTACCCCA
CCTACTAGCTAATCCCATCTGGGCACATCTGATGGCATGAGGCCCGAAGGTCCCC
CACTTTGGTCTTGCGACATTATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCC
CTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCGCTCGTCACCCGAG
AGCAAGCTCTCTGTGCTACCGCTCGACTTGCATGTGTTA

Table 10. Results of sequence data analysis for the KAU-EC1

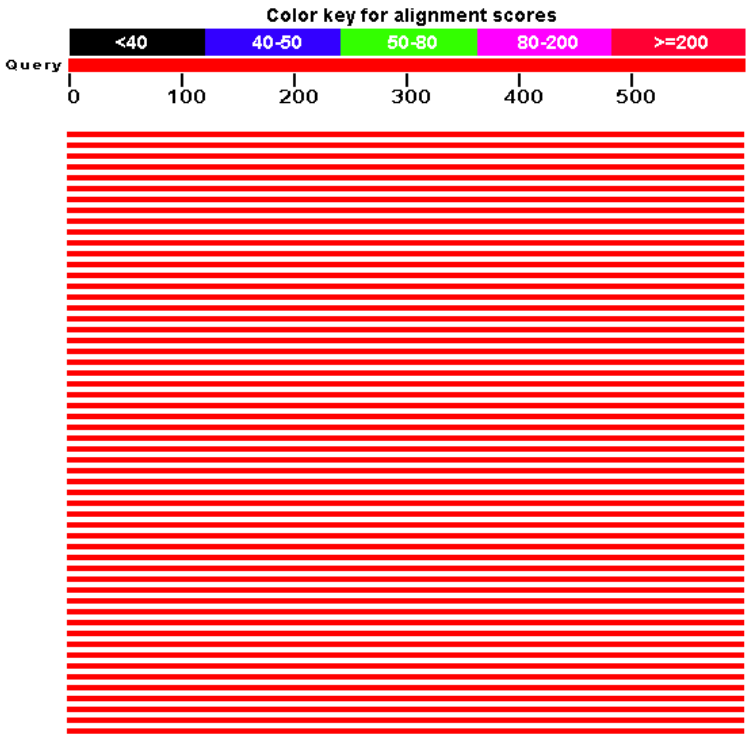
1. Initial sequence length : 646 bp
2. Vector sequence : 10-47 bp
3. Sequence length after Vector/Adapter screening : 609 bp
4. Blastn results

Fig. 2. Results of sequence analysis for the KAU-EC1

Vecscreen output



Blastn output



Accession No.	Description	Q. coverage	E value	Max. ident
EU420956.1	*Klebsiella pneumoniae* strain SA-D6-7 16S ribosomal RNA gene	100 %	0.0	99%

Blastn results indicated homology with *Klebsiella pneumoniae* strain SA-D6-7 16S ribosomal RNA gene. In Blastn results, the query sequence also showed over 99 per cent homology to the *Klebsiella pneumoniae* strain SA-D6-7.

KAU-CC1

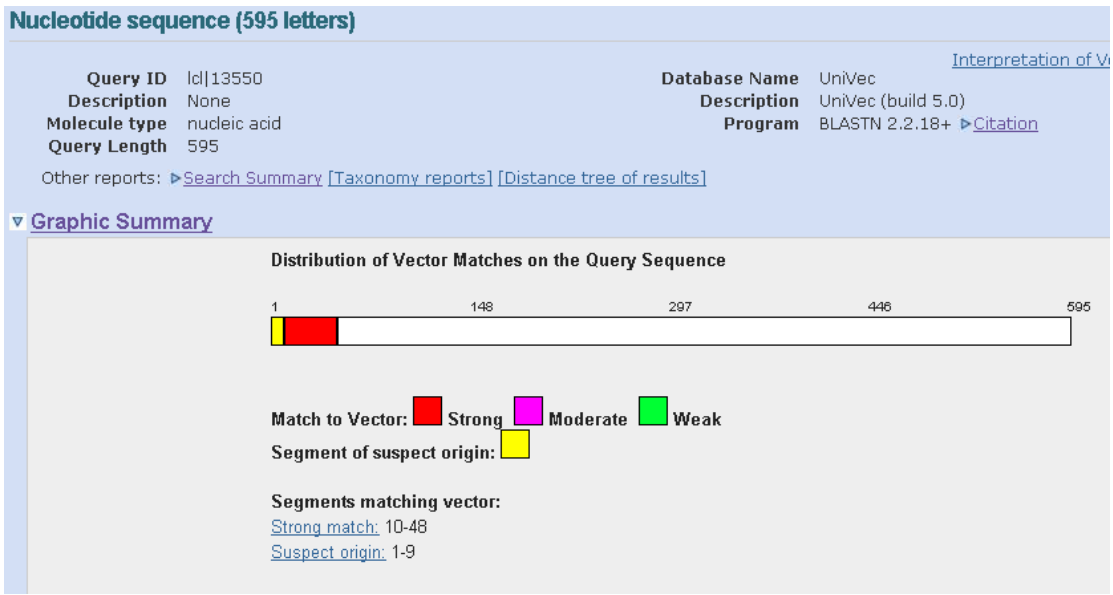
The sequence data obtained for the KAU-CC1 was of 898 bp in size and vector screening indicated vector sequence from 9 to 46 bp. The details of the results obtained are presented in Table 11 and Fig. 3. After vector and adaptor screening, the total sequence obtained for further analysis was 861 bp as detailed below.

Vector/Adapter eluted sequence

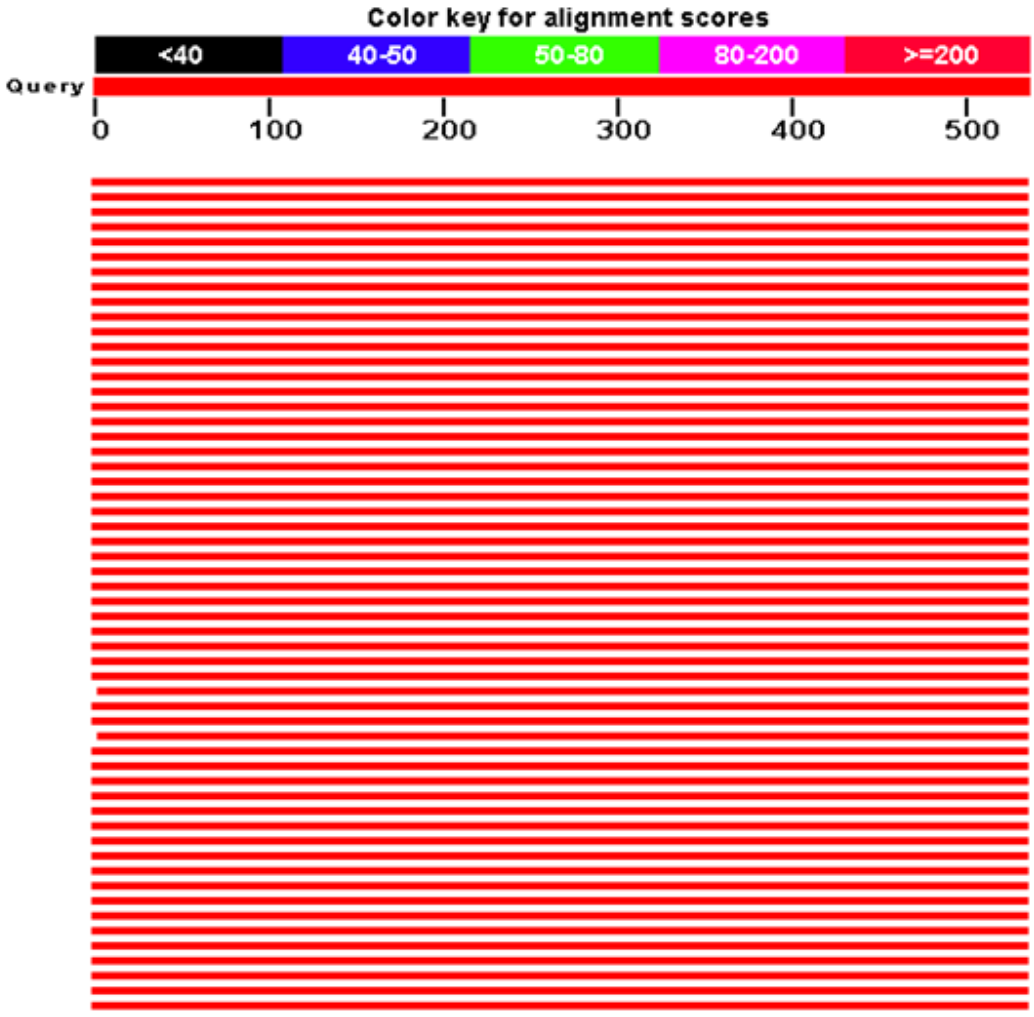
GCGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTCAGGCT
AACACATGCAAGTCGGACGGTAGCACAGAGGAGCTTGCTCCTTGGGTGACGAGT
GGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGATAGAGGGGGATAACCACT
GGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCG
GGCCTCTCACTATCGGATGAACCCAGATGGGATTAGCTAGTAGGCGGGGTAATG
GCCCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGG
AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT
GTAAAGTACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAACCTTGCCGATTG
ACGTTACCCGCAAAGAAGCACCGGCTAACTCCGTGCCACAGCCGCGGTAATACG
GAGGGTGCAAGCGTTAATCGGAATTACTGGGCGGAAACGCACCCAGGCGGTCTG
GCGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTCAGGCT
AACACATGCAAGTCGGACGGTAGCACAGAGGAGCTTGCTCCTTGGGTGACGAGT
GGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGATAGAGGGGGATAACCACT
GGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCG
GGCCTCTCACTATCGGATGAACCCAGATGGGATTAGCTAGTAGGCGGGGTAATG
GCCCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGG
AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT
GTAAAGTACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAA

Fig. 3. Results of sequence analysis for the KAU-CC1

Vecscreen output



Blastn output



CCTTGCCGATTGACGTTACCCGCAAAGAAGCACCGGCTAACTCCGTGCCACAGCC
GCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGGAAACGCACC
CAGGCGGTCTG

Table 11. Results of sequence data analysis for the KAU-CC1

1. Initial sequence length : 595 bp
2. Vector sequence : 10-48 bp
3. Sequence length after Vector/Adapter screening : 557 bp
4. Blastn results

Accession No.	Description	Q. coverage	E value	Max. ident
AY941838.1	*Pantoea agglomerans* strain XW123 16S ribosomal RNA gene	100%	0.0	98%

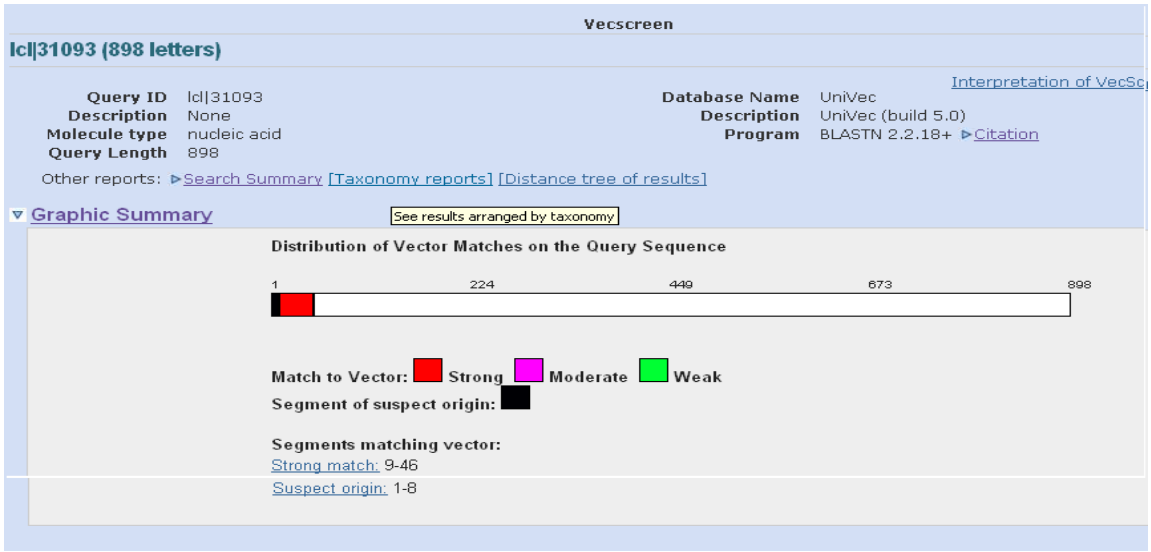
Blastn results indicated homology with *Pantoea agglomerans* strain XW123 16S ribosomal RNA gene. In Blastn results, query sequence also showed over 98 per cent homology to the *Pantoea agglomerans* strain XW12.

KAU-CC2

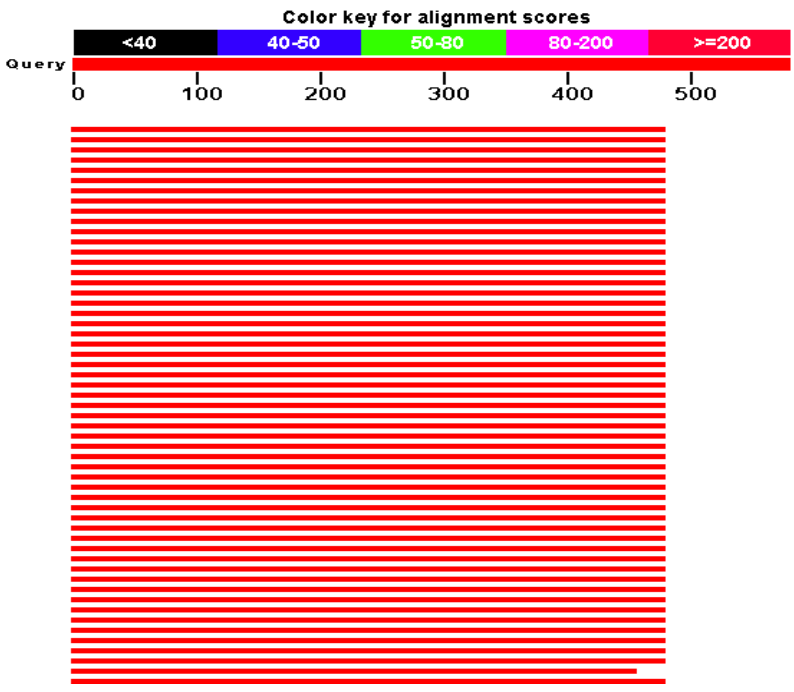
The sequence data obtained for the KAU-CC2 was of 898 bp in size and vector screening indicated vector sequence from 9 to 46 bp. The details of the results obtained are presented in Table 12 and Fig. 4. After vector and adaptor screening, the total sequence obtained for further analysis was 861 bp as detailed below.

Fig. 4. Results of sequence analysis for the KAU-CC2

Vecscreen output



Blastn output



Vector/Adapter eluted sequence

CGATTGGGCCGACGTCGCATGCTCCCGGCCTCATGGCCGCGGGATTGGGCGGAG
TGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGC
GATTCCGACTCCATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTT
TCTGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCTCTGTCCCTACCATTGTAGTA
CGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAACT
AAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAG
CTGACGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGCACCAATCCATCTC
TGGAAAGTTCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATT
AAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGT
CTTGCGACCGTACTCCCAGCGGCGAACTTAACGCGTTAGCTTCGATACTGCGTGC
CAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGCGTGACTACAAGGTATCTA
ATCTGTTTGCTCCCCACGCTTTCTGCCTCATGTCATGTTGGTCAGGTACTGCCTCC
CCATGGAGGTTCCCCTGATTCTACCATTCCTGCTAACAAGAATTCCCTACCCTTAA
CCACCCTAGTGTCAGATACACTGCATTCGGGGTGGGACAAGGTTTCCAGAATTAA
ACACCCCTACCCCCTTAACCGAATTCAACCTGGCCTTTTAACGGGGCGCGCAAAT
AGCGGCTATCTGGGAGG

Table 12. Results of sequence data analysis for the KAU-CC2

5.Initial sequence length : 898 bp

6.Vector sequence : 9-46 bp

7.Sequence length after Vector/Adapter screening : 861 bp

8.Blastn results

Accession No.	Description	Q. coverage	E value	Max. ident
EU221397.1	*Stenotrophomonas maltophila* strain H2S8 16S ribosomal RNA gene	83 %	0.0	95%

Blastn results indicated homology with *Stenotrophomonas maltophila* strain H2S8 16S ribosomal RNA gene. In Blastn results, query sequence also showed over 95 per cent homology to the *Stenotrophomonas maltophila* strain H2S8.

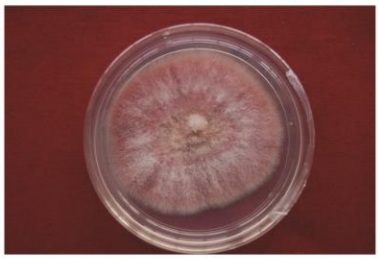
4.1.5.9 Management of endophytic and covert bacteria

Endophytic bacteria (KAU-EC1) was resistant to six antibiotics tested. Covert bacteria (KAU-CC1) managed by adding 50 mg l^{-1} cefotaxime to media. Covert bacteria (KAU-CC2) was resistant to six antibiotics tested.

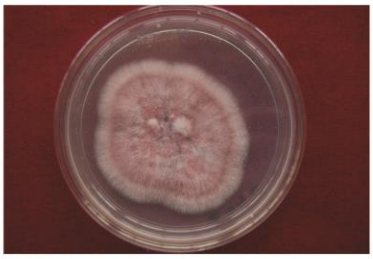
4.2 ISOLATION, IDENTIFICATION AND MANAGEMENT OF ENDOPHYTIC FUNGI FROM NODAL SEGMENTS COLLECTED FROM FIELD PLANTS

Three fungal species were detected from 2, 4 and 6 weeks after culturing of nodal segments derived from field plants. Fungal species were purified by inoculating into PDA media and sent it into National Centre of Fungal Taxonomy, New Delhi for identification of fungi. The fungal species were identified as *Fusarium oxysporum*, *Fusarium moniliformae* and *Botryodiplodia theobromae* (Plate 17).

Contamination with *Fusarium monoliformae*

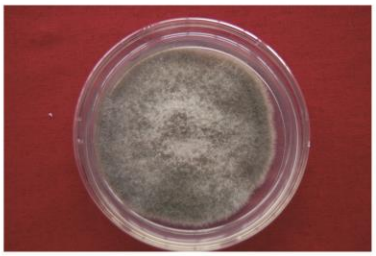


Fusarium monoliformae



Fusarium oxysporum

Contamination with
Botryodiplodia theobromae



Botryodiplodia theobromae

Plate 17. Endophytic fungi isolated from field plants

4.2.1 Management of endophytic fungi

Spraying with 0.1 per cent Bavistin onto field explants and treatment with 0.1% Bavistin for 15 min. before surface sterilization of explants were effective to reduce the contamination. $CuSO_4$ at 100, 150 and 200 mg l^{-1} was added into establishment media. Bavistin at 0.025, 0.05 and 0.1% was also added into different media.

4.3 ESTIMATION OF TOTAL PHENOL

Exudation of phenols is a serious problem for the establishment of cultures derived from field plants (Plate 18,19). The estimation was done according as per Malick and Singh (1980).

4.3.1 Treatments

Total phenol content of the nodal segments of field grown cashew plants were estimated. Total phenol content present in the nodal segments of field grown cashew plant was 8.5 mg g^{-1} of the sample. Standard graph of catechol is given in Fig 5. Phenol leached out into sterile water and liquid media (1/2 MS + 0.4 g glutamine) were estimated at different time periods (Table 13 and Table 14). Friedman two way analysis of variance of ranks was carried out to detect the differential leaching of phenol into liquid media and sterile water. The Friedman test statistic of phenol leached into liquid media was 5.16 at 15.1 per cent level of significance. The Friedman test statistic of phenol leached into sterile water was 14.04 at 0.3 per cent level of significance. The highest mean rank score of phenol leached into liquid media was obtained for 0.2 per cent PVP followed by 0.5 per cent activated charcoal, followed by 100 mg ascorbic acid and control, indicating that 0.2 per cent PVP was more effective. Treatment with ascorbic acid was less effective. The highest mean rank score of phenol leached into sterile water was

Plate 18. Exudation of phenols into the media

Plate 19. Browning of explant

Fig. 5. Standard graph of catochol

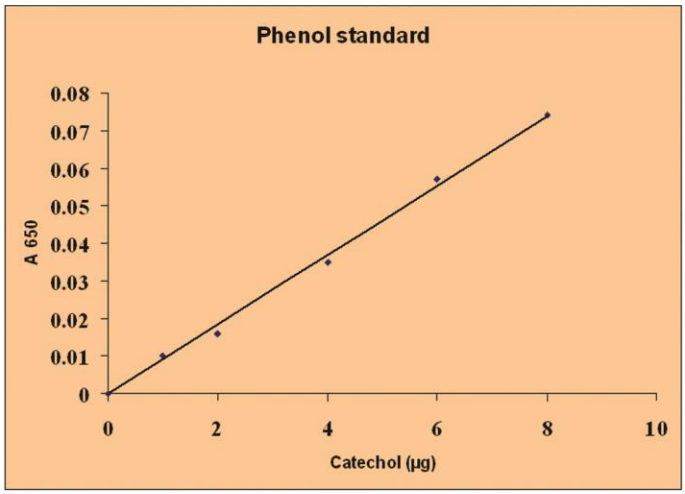


Table 13. Estimation of phenol leached out (mg g^{-1}) into sterile water

Treatments	Phenol content at different time interval			
	15 hrs	**17.5 hrs**	**20 hrs**	**48 hrs**
Control	0.41	0.52	0.59	0.48
0.2% PVP	0.63	0.63	0.63	0.71
100 mg ascorbic acid	0.62	0.62	0.55	0.50
0.5% activated charcoal	0.61	0.62	0.58	0.56

Treatments	Mean rank score
Control	1.88
0.2% PVP	3.75
100 mg ascorbic acid	2.25
0.5% activated charcoal	2.13
Friedman test statistic	5.16
Per cent level of significance	15.1 %

Table 14. Estimation of phenol leached out (mg g^{-1}) into liquid media

Treatments	Phenol content at different time interval (mg g^{-1})				
	15 hrs	**17.5 hrs**	**20 hrs**	**48 hrs**	**53 hrs**
Control	0.37	0.35	0.314	0.33	0.396
0.2% PVP	1.023	1.006	1.05	1.35	1.46
100 mg ascorbic acid	0.33	0.34	0.39	0.29	0.254
0.5% activated charcoal	0.65	0.67	0.45	0.68	0.74

Treatments	Mean rank score
Control	1.80
0.2% PVP	4.00
100 mg ascorbic acid	1.20
0.5% activated charcoal	3.00
Friedman test statistic	14.04
Per cent level of significance	0.3 %

obtained for 0.2 per cent PVP followed by control, followed by 0.5 per cent activated charcoal, and 100 mg ascorbic acid.

4.4 STANDARDIZATION OF *IN VITRO* REGENERATION

4.4.1 EXPLANTS FROM FROM FIELD PLANTS

4.4.1.1 Standardization of surface sterilization

Nodal segments were taken from field grown cashew plants. To standardize surface sterilization of explants $HgCl_2$ at varying concentration (0.05 and 0.1%) were tried at varying time intervals. Effect of various concentrations of $HgCl_2$ on surface sterilization is represented in Fig. 6.

For nodal segments 0.1 per cent $HgCl_2$ for 2 minutes proved to be the best. Higher concentration of $HgCl_2$ resulted in yellowing of the explants.

4.4.1.2 Effect of basal media on regeneration

To study the effect of basal media on regeneration, the nodal segments were inoculated to MS, ½ MS media. The regeneration of nodal explants was observed in MS and ½ MS media but ½ MS media was found to be the best. Forty five per cent bud sprouting was observed in ½ MS media whereas twenty per cent bud sprouting was observed in full MS media. Effect of basal media on bud sprouting is given in Fig. 7.

4.4.1.3 Effect of season on survival and bud break of cashew explants

Explants were collected from January to December 2007. Number of explants varied between months depending on their availability. Survival per cent was high during dry months (March-May) and low during rainy season

Figure 6. Effect of various concentrations of 0.1% $HgCl_2$ and time of sterilization on survival of nodal segments derived from field grown cashew plants

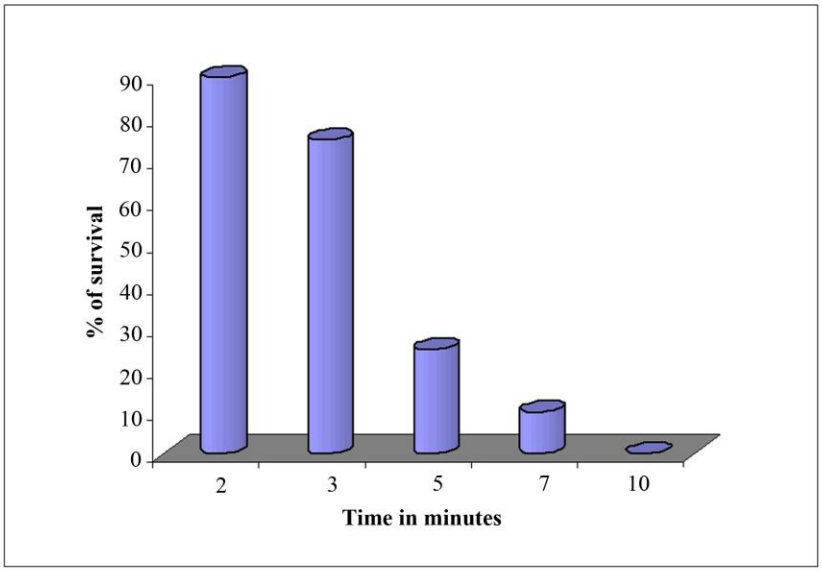
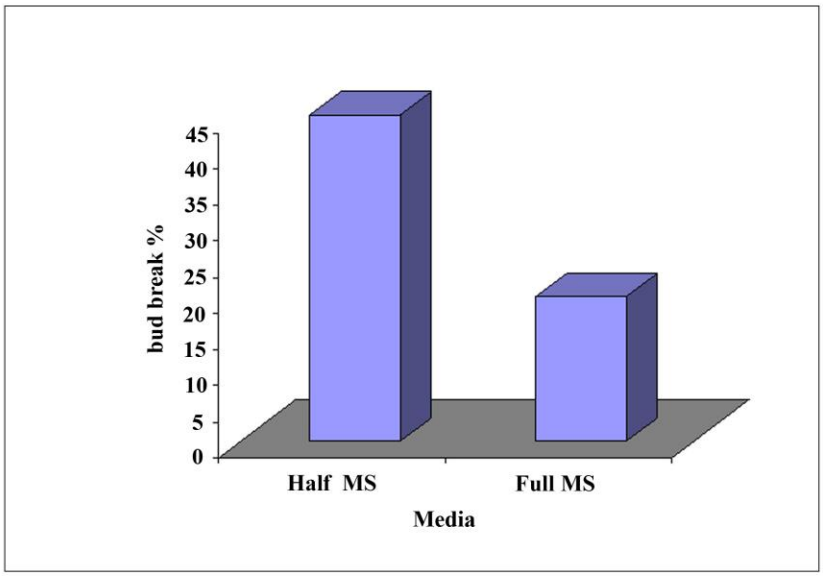


Figure 7. Effect of basal media on nodal cultures



(June-October). On the other hand, bud break was low during the rainy season and high during dry months. Effect of season on survival and bud break of cashew explants after 5-6 weeks culturing are shown in the Fig. 8.

4.4.1.4 Culture establishment

Cultures of nodal segments and shoot tips were established on media within 7 days. The combination ½ MS + 400 mgl^{-1} Glutamine +0.2% PVP produced good establishment. Bud burst occurred within 7 days (Plate 20). Effect of different media composition on establishment of nodal culture is shown in Table 15.

4.4.1.5 Multiplication and elongation

Multiple shoots from nodal segments initiated within 15 days after inoculation. Swelling of the basal part of the nodal segments occurred prior to multiple shoot formation. The combination ½ MS + 400 mgl^{-1} Glutamine +0.2% PVP+0.45 µM TDZ produced maximum shoot buds with mean value of 3 (Plate 21). The effect of different media composition on multiplication and elongation are shown in Table 16.

4.4.1.6 Rooting

Elongated shoots failed to produce roots in half MS + 1 mg l^{-1} IAA+ 1 mg l^{-1} IBA. Pulse treatment with 24 ppm IBA was also carried out for 24 hrs before inoculation of elongated shoots in media. Effect of different concentrations of auxins tried for rooting is shown in Table 17.

Plate 20. Bud burst

Plate 21. Formation of multiple shoots

Figure 8. Effect of season on survival and bud break of cashew explants after 5-6 week of culturing

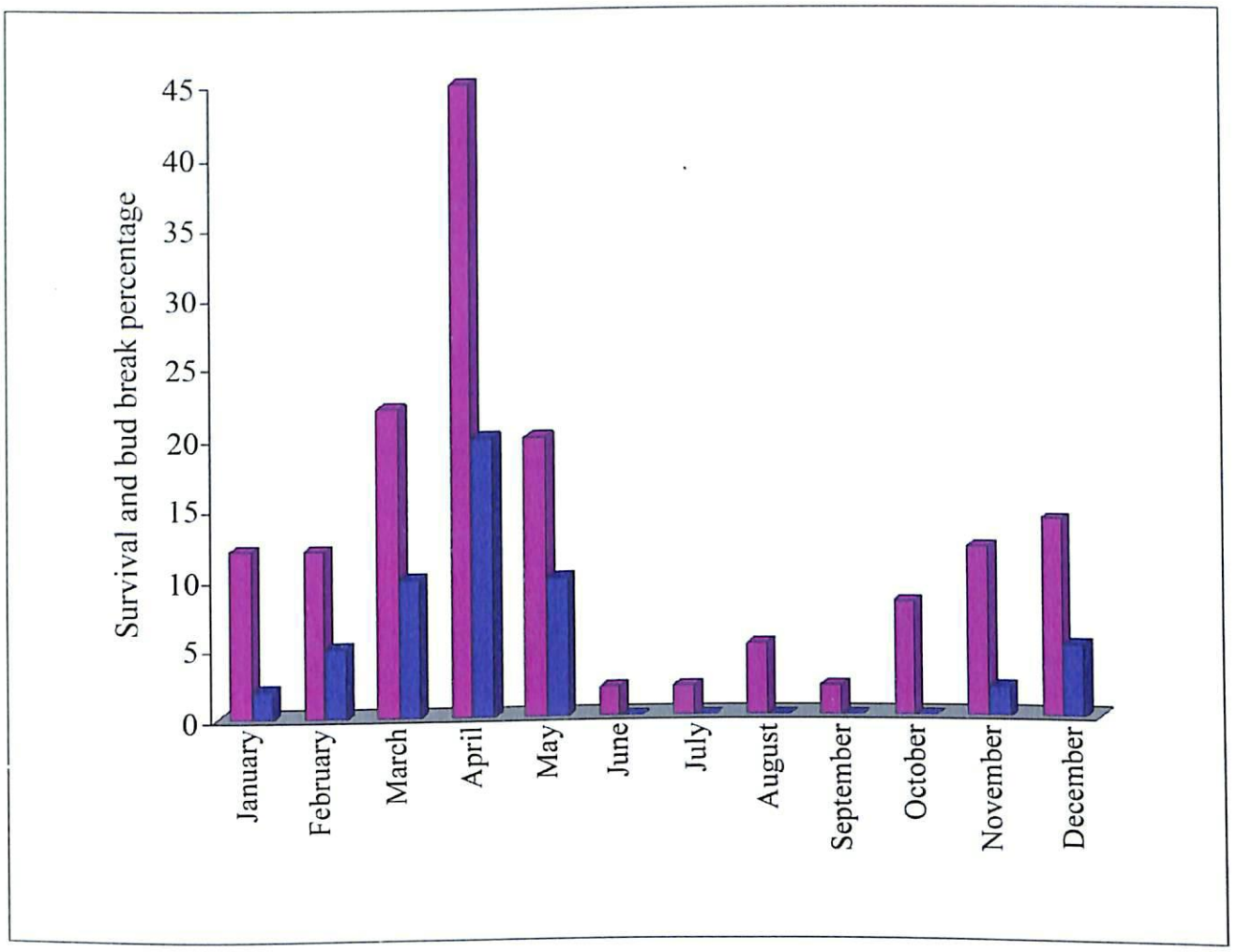


Table 15. Effect of different media composition on establishment of nodal cultures

Media	Number of days for bud burst	Sprouting (%)
½ MS + 400 mgl^{-1} glutamine+0.2% PVP	**7**	20
½ MS + 1 mg l^{-1} BA + 1 mg l^{-1} IAA+0.2% PVP	25	8
½ MS + 1 mg l^{-1} BA + 1 mg l^{-1} NAA+0.2% PVP	36	5

Table 16. Effect of different media composition on multiplication and elongation

Media	Number of multiple shoot bud/node	Number of elongated shoot bud/node	Length (cm)
½ MS + 400 mgl^{-1} glutamine + 0.45 µM TDZ+0.2% PVP	3	1	2
½ MS + 1 mg l^{-1} BA + 0.5 mg l^{-1} IAA+0.2% PVP	1	1	0.5
½ MS + 1.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA+0.2% PVP	1	1	0.3
½ MS + 1.5 mg l^{-1} BA + 1 mg l^{-1} IAA+0.2% PVP	1	1	0.8

Table 17. Effect of different concentrations of auxins tried for rooting of elongated shoots derived from field grown plants

Media	Culture responding (%)
½ MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA	0
½ MS + 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IAA	0
½ MS + 1 mg l^{-1} NAA + 0.5 mg l^{-1} IAA	0
½ MS + 1 mg l^{-1} NAA + 0.5 mg l^{-1} IBA	0
½ MS + 1 mg l^{-1} NAA + 1 mg l^{-1} IAA	0
½ MS + 1 mg l^{-1} NAA + 1 mg l^{-1} IBA	0
½ MS + 0.5 mg l^{-1} IBA + 0.5 mg l^{-1} IAA	0
½ MS + 0.5 mg l^{-1} IBA + 1 mg l^{-1} IAA	0
½ MS + 1 mg l^{-1} IBA + 0.5 mg l^{-1} IAA	0
½ MS + 1 mg l^{-1} IBA + 1mg l^{-1} IAA	0
½ MS + 1.5 mg l^{-1} IBA + 1mg l^{-1} IAA	0
½ MS + 1.5 mg l^{-1} IBA + 1.5 mg l^{-1} IAA	0

4.4.2 *IN VITRO* SEED GERMINATION

The seeds were soaked in water for 4 days and germinated *in vitro* on full MS medium with different concentration of growth regulators. Mature nuts were used to raise *in vitro* seedlings. The embryo with the radicle was excised carefully (Plate 22) and inoculated into MS medium with different growth regulators (Plate 23). The effect of different concentrations of growth regulators on *in vitro* seed germination are shown in Table 18. The maximum seed germination (80 %) was obtained on MS + 3.5 gl^{-1} kinetin + 1 mgl^{-1} NAA which was followed by MS + 2.5 mgl^{-1} kinetin + 0.5 mgl^{-1} NAA which recorded 25 per cent germination. The seeds were germinated within 15 days after inoculation in the former case. By 4 weeks, 8-10 cm long seedlings were obtained. In the later case, seeds were germinated within 30 days after inoculation. By 6 weeks 8-10 cm long seedlings were obtained. Healthy seedlings were obtained on the both the media (Plate 24, 25).

4.4.3 EXPLANTS FROM *IN VITRO* SEEDLINGS

4.4.3.1 Culture establishment and multiplication

Cultures of cotyledonary nodes, nodal segments and shoot tips were established on media within 7 days (Plate 26). The effect of different concentrations of growth regulators on establishment and multiplication from cotyledonary nodes, nodal segments and shoot tips are shown in Table 19.

Multiple shoots from cotyledonary nodes, nodal segments and shoot tips initiated within 10 days after inoculation. Swelling of the basal part of the cotyledonary nodes, nodal segments and shoot tips occurred prior to multiple shoot formation.

Plate 22. Embryo of cashew

Plate 20. Elongation of radicle at the proximal end of cotyledon

Plate 21. *In vitro* seedling

Plate 22. Nodal segments of *in vitro* seedlings as explants

Plate 26. Establishment of explants

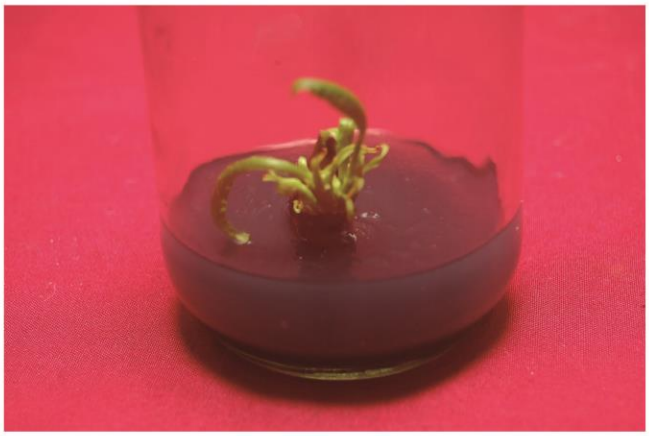


Plate 27. Multiple shoots derived from cotyledonary node of cashew (MS 1.5 mg l^{-1} BA+IAA 1 mg l^{-1})

Table 18. Effect of different concentrations of growth regulators on *in vitro* seed germination in MS medium

MS+growth regulators (mgl^{-1})+0.05 % activated charcoal		Seed germination (%)	No.of days taken for germination
Kinetin	NAA		
2.5	0.5	25	30 days
3.5	1	80	15 days
0	0	10	52 days

Each treatment consists of 200 replicates, observation after 30 days.

Table 19. Effect of different concentrations of growth regulators on establishment and multiplication of shoot buds from cotyledonary nodes, nodal segments and shoot tips in MS medium

MS+Growth regulators (mgl^{-1})		Cotyledonary nodes			Nodal segments			Shoot tips		
BA	IAA	No. of multiple shoot buds/node	No. of elongated shoot buds/node	Length (cm)	No. of multiple shoot buds/node	No. of elongated shoot buds/node	Length (cm)	No. of multiple shoot buds/node	No. of elongated shoot buds/node	Length (cm)
1	0.5	2.9	1	0.5	1.6	1	0.6	1	1	0.4
1.5	1	3.4	2	1.2	2.3	2	1.4	2	1	0.9
2	1	2.3	2	1.2	2	2	2	1.4	1	1
2.5	1	2.2	2	2	2.2	1	3	1	1	2
3	1.5	2.2	1	2	2	1	2	1	1	0.8

Each treatment consists of 20 replicates, observation after 30 days.

Eighty per cent regeneration response was obtained from nodal segments, cotyledonary nodes and shoot tips in all the concentrations of BA and IAA tested. The combination MS+1.5 mgl^{-1} BA+1 mgl^{-1} IAA produced maximum shoot buds per cotyledonary nodes, nodal segments and shoot tip with a mean value of 3.4 respectively (Plate 27). Taking the shoot buds and elongation together maximum multiplication was obtained from MS + BA 2.5 mgl^{-1} + IAA 1 mgl^{-1} (Plate 28).

Compared to shoot tips and nodal segments, cotyledonary nodes produced more multiple shoots (upto 5). The number of elongated shoots was more at 2.5 mg l^{-1} BA.

4.4.3.2 Elongation

Elongation occurred in all the media combinations tried. Data regarding the elongation of cotyledonary nodes and nodal segments in different culture medium is given in Table 20.

Maximum elongation was obtained on MS+ 2 mgl^{-1} BA + 400 mgl^{-1} glutamic acid where the mean length 2.5 cm was obtained. The number of internodes and leaves were also highest in this combination. The plantlets showed maximum length and vigor with dark green leaves in this medium (Plate 29, 30). The combination MS + 2.5 mg l^{-1} BA + 400 mg l^{-1} glutamic acid produced seedlings with a mean length of 1.48 cm and 5 large leaves which was followed by MS with 2.5 mgl^{-1} BA that produced mean length of 1.57 cm and 4 leaves.

4.4.3.3 Rooting

Eighty per cent rooting occurred in half MS + sucrose 3 per cent, 1 mgl^{-1} IBA and 1 mgl^{-1} IAA with 4.3 as the mean number of roots. Pulse treatment with IBA before inoculation of elongated shoots was carried out at different

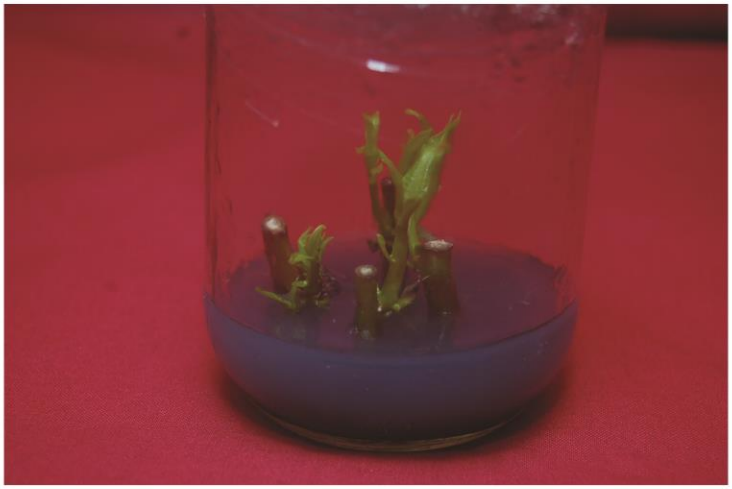


Plate 28. Multiple shoots derived from nodal segments of cashew (MS + 2.5 mg l^{-1}BA + 1 mg l^{-1} IAA)

Plate 29. Elongated shoots derived from cotyledonary node of cashew

Plate 30. Elongated shoots derived from nodal segments of cashew

Table 20. Elongation of shoot buds derived from nodal segments, cotyledonary nodes in different medium

Media	Length of shoot (cm)	No. of internodes/shoot	No. of leaves/shoot
MS+2 mgl^{-1} BA + 400 mgl^{-1} glutamic acid	2.55	1.42	5
MS+2.5 mgl^{-1} BA + 400 mgl^{-1} glutamic acid	1.57	1	4
MS+3 mgl^{-1} BA + 400 mgl^{-1} glutamic acid	1.48	1	5
MS+400 mgl^{-1} glutamic acid	0.5	1	3

Table 21. Rooting of shoot buds derived from nodal segments, cotyledonary nodes on MS medium supplemented with different auxin concentrations after pulse treatment with IBA

Media	Culture responding (%)	Average number of roots / shoots	Average root length (cm)
¼ MS + 200 mg l^{-1} glutamic acid + 1 mg l^{-1} IBA	0		
¼ MS + 200 mg l^{-1} glutamic acid + 1.5 mg l^{-1} IBA	0		
¼ MS + 200 mg l^{-1} glutamic acid + 2 mg l^{-1} IBA	0		
¼ MS + 200 mg l^{-1} glutamic acid + 1 mg l^{-1} IBA + 1 mg l^{-1} IAA	0		
½ MS + 0.5 mg l^{-1} IBA + 0.5 mg l^{-1} IAA	0		
½ MS +1 mg l^{-1} IBA+ 0.5 mg l^{-1} IAA	0		
½ MS +1 mg l^{-1} IBA + 1 mg l^{-1} IAA	80	4.3	4.38
½ MS + 0.5 mg l^{-1} IBA +1 mg l^{-1} IAA	0		

concentration. Rooting occurred in pulse treatment with 24ppm IBA for 24 hrs. This combination produced both main and lateral roots with a greater mean length of 4.38 cm (Plate 31, 32). Slight callusing occurred at the basal portion of a few shoots. The leaves were larger and greenish. Data regarding number of roots, length of roots in different culture media are given in Table 21.

4.4.3.3.1 Effect of *Glomus fasciculatum* on *in vitro* rooting

The procedure reported by Gerdemann and Nicolson (1963) was used for the isolation of AM fungal spores from the rhizosphere soil. Spores after surface sterilization were inoculated into solid media before the initiation of rooting and after the initiation of rooting. Per cent root colonization was assessed by using the method described by Phillips and Hayman (1970). There was no colonization upto 30 days.

4.4.3.3.2 Effect of *Bacillus subtilis* on *in vitro* rooting

Twenty four hrs old *Bacillus subtilis* culture was used as inoculum (Plate 33). Bacterial inoculum (10^8 cfu) was added into the media at two stages (1) after the initiation of roots (2) three days before transplanting. Basal portion of explants decayed after one week due to the higher multiplication rate of bacteria.

4.4.3.3.3 Root induction with *Agrobacterium rhizogenes*

4.4.3.3.3.1 Culturing and sensitivity screening of *Agrobacterium* and explants

4.4.3.3.3.1.1 Culturing of *A. rhizogenes* strains

The *A. rhizogenes* were cultured on YEB, YEM, NA and LBA media. The strains differed in their growth on the four media tested. The influence of culture media on the growth of *A. rhizogenes* strains is given in Table 22.

Plate 31. Initiation of roots

Plate 32. Rooting of shoots derived from from elongated shoot buds of cashew

Plate 33. *Bacillus subtilis*

Table 22. Influence of culture media on the growth of *A. rhizogenes* strains

***A. rhizogenes* strains**	**YEB**	**YEM**	**NA**	**LBA**
MTCC 532	+ +	+ +	+ +	+ +
MTCC 2364	+ + +	+ +	+ + +	+ + +

+ slow growth, + + fast growth, + + + very fast growth

All the culture media favoured the growth of *A. rhizogenes* strains. Strain MTCC 2364 showed faster growth in all the media except YEM. So YEM was selected for growing MTCC 2364, so as to obtain single colonies. YEB and NA were preferred for growing MTCC 532 and MTCC 2364.

4.4.3.3.3.1.2 Cultural characteristics of *Agrobacterium*

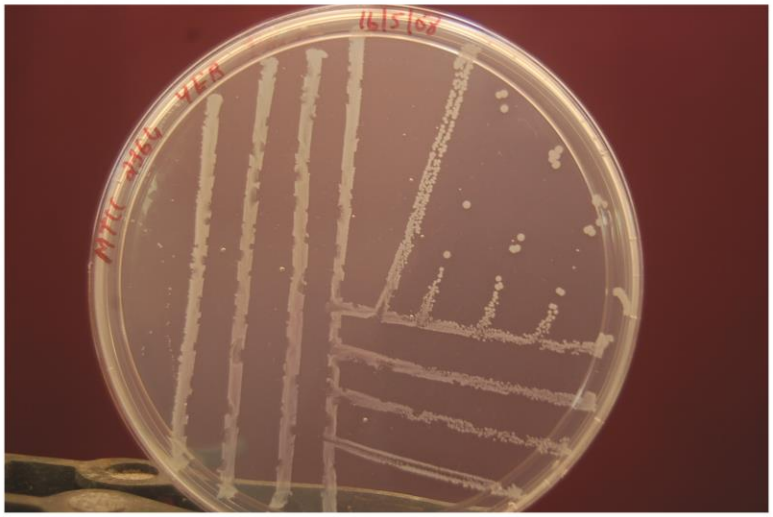
The colonies of strain MTCC 2364 appeared within one day and the colonies were of larger size with seated margin and were whitish in colour. The strain MTCC 532 produced smooth round small colonies at closer spacing two days after streaking and were whitish and mucoid in nature (Plate 34).

4.4.3.3.3.1.3 Screening of *A. rhizogenes* strains for antibiotic sensitivity

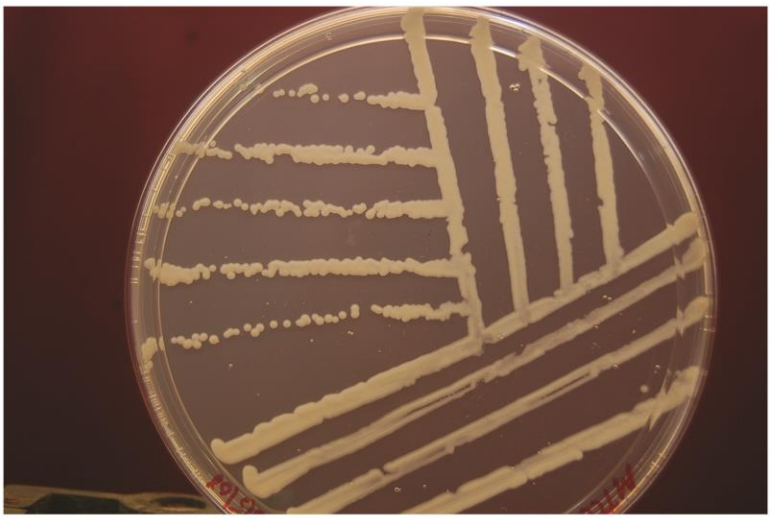
The response of *A. rhizogenes* strains to different concentrations of antibiotics such as ampicillin, cefotaxime and carbencillin is given in Table 23.

The strain MTCC 2364 showed resistance to both ampicillin and carbencillin upto 400 mg l^{-1}. MTCC 532 was found to be sensitive to carbencillin. Both strains were sensitive to cefotaxime and ampicillin at 500 mg l^{-1}.

The strain MTCC 2364 and MTCC 532 survived upto 400 mg l^{-1} cefotaxime. Cefotaxime at 500 mg l^{-1} killed all the two strains of *A. rhizogenes*



MTCC 2364



MTCC 532

Plate 34. ***Agrobacterium rhizogenes*** **strains employed in the study**

Table 23. Sensitivity of *Agrobacterium* strains to different antibiotics

Antibiotics	Concentration (mg l^{-1})	Response of *Agrobacteriumrhizogenes* strains	
		MTCC 2364	MTCC 532
Ampicillin	0	+ +	+ +
	50	+ +	+
	100	+ +	+
	200	+ +	+
	300	+ +	+
	400	+ +	+
	500	-	-
Carbencillin	0	+ +	+ +
	50	+ +	-
	100	+ +	-
	200	+ +	-
	300	+ +	-
	400	+ +	-
	500	+ +	-
Cefotaxime	0	+ +	+ +
	50	+	+
	100	+	+
	200	+	+
	300	+	+
	400	+	+
	500	-	-

- No growth, + restricted growth, + + good growth

-

(Plate 35). So 500 mg l^{-1} cefotaxime was taken as the optimum concentration of antibiotic to kill *A. rhizogenes* strains under study.

4.4.3.3.3.1.4 Sensitivity of explants to antibiotics

The sensitivity of explants to ampicillin and cefotaxime at different concentrations is depicted in Table 24. Shoot tips were found to be healthy in 500 mg l^{-1} ampicillin and 500 mg l^{-1} cefotaxime. All the explants were pale at 600 mg l^{-1}, 700 mg l^{-1} ampicillin and cefotaxime.

4.4.3.3.3.2 Standardization of inoculation methods

4.4.3.3.3.2.1 Direct inoculation and suspension culture method

4.4.3.3.3.2.1.1 Influence of bacterial inoculum

.

Both the single cell colonies and bacterial suspension produced rooting. The strain MTCC 2364 showed rooting (30 per cent) by both methods. The strain MTCC 532 did not induce roots in shoot tips.

4.4.3.3.3.2.1.2 Influence of co-culture periods

The explants after infection were co-cultured for 1-3 days at 26 +/- 2.0^{o} C under dark. Slight bacterial ooze or bacterial growth appeared around each explant on co-cultivation for 1-2 days. Normally bacterial overgrowth occurred on third day of co-cultivation, which can be removed by washing and further culturing the explants in antibiotics containing culture media. In due course of cultivation, no signs of explants necrosis were seen and the explants remained healthy and green.

The transformation frequency was influenced by co-culture period. Rooting occurred in explants co-cultivated for 3 days.

Plate 35. Evaluation of sensitivity of MTCC 532 and MTCC 2364 to various concentrations of Cefotaxime

Table 24. Screening of explant sensitivity to different antibiotics

Antibiotics	Concentration ((mg ml^{-1})	Response	Results
Cefotaxime	0	Healthy	Resistant
	100	Healthy	Resistant
	200	Healthy	Resistant
	300	Healthy	Resistant
	400	Healthy	Resistant
	500	Healthy	Resistant
	600	Pale	Sensitive
	700	Pale	Sensitive
Ampicillin	0	Healthy	Resistant
	100	Healthy	Resistant
	200	Healthy	Resistant
	300	Healthy	Resistant
	400	Healthy	Resistant
	500	Healthy	Resistant
	600	Pale	Sensitive
	700	Pale	Sensitive

4.4.3.3.3.3 Effect of acetosyringone

The acetosyringone at concentrations of 10 mM and 20 mM were incorporated into media containing antibiotics. With the strain MTCC 532, MTCC 2364 the presence of acetosyringone was not found to positively influence rooting percentage. No transformation was obtained from shoot tips in the presence of acetosyringone.

4.4.3.3.3.4 Efficiency of strains in inducing roots

The strain MTCC 2364 showed 30 per cent rooting. The strain MTCC 532 failed to produce roots (Plate 36).

4.4.3.3.3.5 Number of days for root induction

Twenty five days were taken for root induction with the strain MTCC 2364.

4.5 HARDENING AND PLANTING OUT

The rooted plantlets were transferred to pots containing sterile sand (Plate 37, 38). Four treatments were carried out at hardening stage i.e. inoculation with *Glomus fasciculatum*, *Bacillus subtilis*, their consortium and control (Plate 39).

4.5.1 EFFECTS OF *GLOMUS FASCICULATUM* AND *BACILLUS SUBTILIS* ON SURVIVAL AND GROWTH OF MICROPROPAGATED CASHEW PLANTS

Inoculation with *Glomus fasciculatum*, *Bacillus subtilis* and both combinations had influenced the survival rate and plant growth characteristics

A. Rooting with *Agrobacterium rhizogenes*

B. Control

Plate 36. Rooting with *Agrobacterium rhizogenes*

Plate 37. Rooted plant

Plate 38. Hardening

Plate 39. Hardened plants with various treatments

such as plant height, number of leaves and leaf area of tissue culture derived plantlets of cashew.

4.5.1.1 Effects of *Glomus fasciculatum* and *Bacillus subtilis* on survival of micropropagated cashew plants

The survival rate of the plantlets was found to improve due to inoculation with *Glomus fasciculatum* and *Bacillus subtilis*. There was not much mortality of plantlets till 20 DAT (Days After Transplanting). Considerable mortality was noticed from 20 DAT-50 DAT in control. i. e. 25.8 per cent, 38.5 per cent, 39.57 per cent, 48.52 per cent and 49.72 per cent mortality was noticed in 25 DAT, 30 DAT, 40 DAT, 50 DAT and 60 DAT respectively.

The treatment with *Glomus fasciculatum* and *Bacillus subtilis* showed 70.72 per cent survival after 20 DAT. The treatment with *Bacillus subtilis* alone showed 68.35 per cent survival and the treatment with *Glomus fasciculatum* alone showed 66.68 per cent survival. Control recorded 50.22 per cent survival. There was not much mortality after 60 DAT. The treatment involving *Glomus fasciculatum* and *Bacillus subtilis* and *Bacillus subtilis* alone showed remarkably higher survival rate compared to control and *Glomus fasciculatum* alone. Observations recorded on survival of micropropagated plants at 30^{th}, 60^{th} and 90^{th} day of planting out after inoculation with *Glomus fasciculatum* and *Bacillus subtilis* are presented in Table 25.

4.5.1.2 Effect of *Glomus fasciculatum* and *Bacillus subtilis* on height of tissue culture plantlets of cashew

The observations recorded on plant height at 30^{th}, 60^{th}, 90^{th} and 120^{th} day of planting out after inoculation with *Glomus fasciculatum* and *Bacillus subtilis* were presented in Table 26.

Table 25. Effect of *Glomus fasciculatum* and *Bacillus subtilis* on survival of tissue culture plantlets of cashew

Treatments	Number of plants survived (%)					
	20 DAT	**30 DAT**	**40 DAT**	**50 DAT**	**60 DAT**	**90 DAT**
GB_0	66.68	66.68	66.68	66.68	66.68	66.68
G_0B	68.35	68.35	68.35	68.35	68.35	68.35
GB	70.72	70.72	70.72	70.72	70.72	70.72
G_0B_0	74.2	61.5	60.43	51.48	50.22	50.22

GB_0 – Treatment with *Glomus fasciculatum*

G_0B -Treatment with *Bacillus subtilis*

GB - Treatment with *Glomus fasciculatum* and *Bacillus subtilis*

G_0B_0 – Control

DAT- Days after transplanting

Treatments	Mean rank score
GB_0	1.83
G_0B	2.83
GB	3.83
G_0B_0	1.5
Friedman test statistic	12
Per cent level of significance	0.7%

Table 26. Effect of microbial inoculants on height (cm) of tissue cultured plantlets of cashew

Treatments	30 DAT	60 DAT	90 DAT	120 DAT
GB_0	8.5	9.2	9.6	11.2
G_0B	7.5	8.5	9.2	11
GB	8.8	9.4	10.2	12
G_0B_0	7.7	8.5	8.9	9

DAT- Days after transplanting

The combination of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* showed further increase in plant height of 12 cm on the 120th day which was superior over *Bacillus subtilis* and *Glomus* alone. Treatment with *Glomus fasciculatum* showed maximum plant height which was 11.2 cm over that for *Bacillus subtilis*. The combination treatment of *Glomus fasciculatum* and *Bacillus subtilis* consistently showed higher plant height compared to individual inoculations and control treatment. The treatment recorded plant height of 8.5, 9.2, 9.6, 11.2 cm for *Glomus fasciculatum* alone and 7.5, 8.5, 9.2, 11 cm with *Bacillus subtilis* alone on 30th, 60th, 90th and 120th day of planting out respectively. Dual inoculation of *Bacillus subtilis* and *Glomus fasciculatum* showed height of 8.8, 9.4, 10.2, 12 cm on 30 DAT, 60 DAT, 90 DAT and 120 DAT. This combination treatment was found to be most effective which recorded 12 cm on 120th day of planting out. This treatment was significant over control (9 cm), individual inoculation of *Bacillus subtilis* (11 cm), *Glomus fasciculatum* (11.2 cm) (Plates 40, 41, 42, 43).

4.5.1.3 Effect of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* on leaf formation in tissue culture plantlets of cashew

Effect of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* on leaf formation in tissue culture plantlets of cashew is given in Table 27. There was not much difference in the number of leaves between the treatments. The maximum number of leaves was shown by treatment 6 on 60th, 7 on 90th and 8 on 120th day of planting out whereas the control recorded 5.45 leaves on 60th, 7 leaves on 90th and 8 leaves on 120th day after transplanting respectively (Plates 40, 41, 42, 43).

Plate 40. 30 Days after transplanting

Plate 41. 90 days after transplanting

Plate 42. 120 days after transplanting

Treatment with *Glomus fasciculatum*

Treatment with *Glomus fasciculatum and Bacillus subtilis*

Treatment with *Bacillus subtilis*

Plate 43. Effect of various treatments on plant growth

Table 27. Effect of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* on leaf formation in tissue culture plantlets of cashew

Treatments	Number of leaves			
	30 DAT	60 DAT	90 DAT	120 DAT
GB_0	4	6	7	8
G_0B	3	6	7	8
GB	4	6	7	8
G_0B_0	4.1	5.45	7	8

Table 28. Mycorrhizal colonization in tissue culture plantlets of cashew inoculated with *Glomus fasciculatum*, *Glomus fasciculatum* and *Bacillus subtilis* consortium

Sl.No.	Age of the plantlet (days)	Colonization (%)	Presence of 550 bp PCR product
1	45	30	+
2	60	50	+
3	90	70	+

4.5.1.4 Effect of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* on root growth in tissue culture plantlets of cashew

The observations recorded on root growth at 120th day of planting out after inoculation with *Glomus fasciculatum* and *Bacillus subtilis*. The combination of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* showed maximum root growth of 7 cm on the 120th day which was superior over *Bacillus subtilis* and *Glomus* alone. Treatment with *Glomus fasciculatum* showed root growth of 5.5 cm and treatment with *Bacillus subtilis* showed root growth of 4 cm and 3 cm for control (Plate 44).

4.5.2 DETECTION OF AN ARBUSCULAR MYCORRHIZAL FUNGUS IN ROOTS OF TISSUE CULTURE DERIVED CASHEW PLANTS

The 550 bp rDNA amplification product characteristic of AM fungi was consistently amplified from roots of tissue cultured plantlets colonized by *Glomus fasciculatum*. The AM fungus was detected in roots after 45 days, when the colonization was at least 30 per cent (Plate 45). Colonization was 70 per cent after 90 days (Table 28). The extent of root colonization and not the harvest date appeared to be critical for detection. *Glomus fasciculatum* was also isolated from the sand that used for transplanting the plantlets (Plate 46).

The 550 bp was not detected in the first amplification. The PCR product was detected only after two amplification rounds of 40 cycles. Plate 47 shows typical PCR results obtained with non-colonized and colonized roots. The 550 bp product was never detected from uninoculated, non-colonized control plants, even after two amplification rounds of 40 cycles each.

The addition of Chelex resin to the Tris-HCl buffer when the colonized roots were boiled resulted in increased amplification of the 550 bp AM

Glomus fasciculatum

Glomus fasciculatum and *Bacillus subtilis*

Bacillus subtilis

Control

Plate 44. Effect of root growth at different treatments

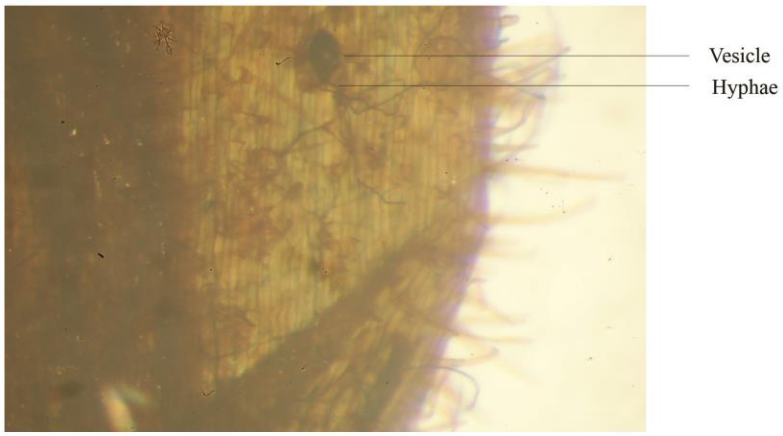


Plate 45. Colonization of AM fungus in tissue culturally derived cashew plants

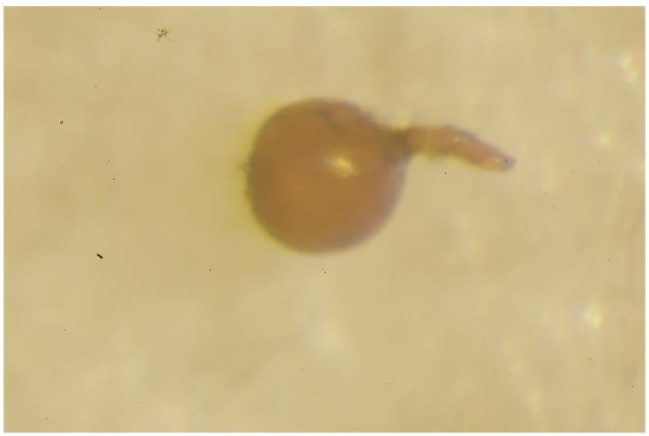
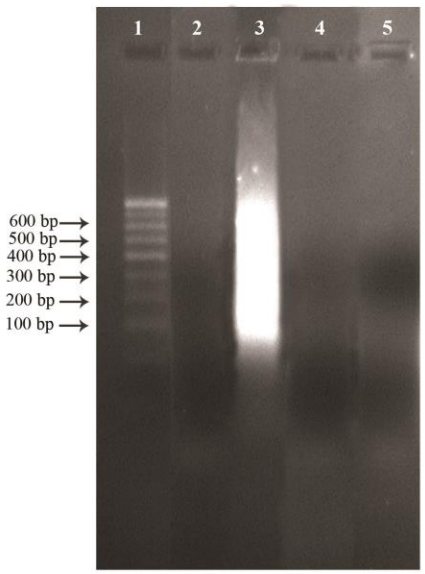


Plate 46. Spore of *Glomus fasiculatum*



Lane 1 - 100 bp marker
Lane 2 - PCR product of first amplification
Lane 3 - Reamplified PCR product
Lane 4 - Control
Lane 5 - Blank

Plate 47. Amplification of 18S rDNA with VANSI and NS21

rDNA product from reaction mixture. Dilution of the template DNA was done and 1, 5 and 10 fold dilutions which provided positive results.

Discussion

DISCUSSION

Cashew is an important cash crop cultivated in the tropical regions of India, Brazil and Africa. Because of its hardy and drought resistant nature, it is grown in a wide variety of soils under very low management conditions. It is propagated mainly by seeds and this often results in high levels of variability (Philip and Unni, 1984). Conventional vegetative propagation methods, e.g. air layering, grafting or cuttings are not sufficiently rapid, and techniques like micropropagation via multiple axillary branching and *in vitro* organogenesis or embryogenesis offer prospects of faster multiplication of elite genotypes. But cashew, like other Anacardiaceae, is strongly recalcitrant to *in vitro* culture and only limited successes have been achieved (Philip, 1984; Jha, 1988; Leva and Falcone, 1990; Mantell *et al.*, 1998; Keshavachandran, 1996; Keshavachandran and Shelja, 1999; Keshavachandran, 2004; Keshavachandran and Riji, 2005).

Progress with application of micropropagation of cashew has been achieved using microshoots (Lievens *et al.*, 1989; Leva and Falcone, 1990), cotyledonary nodes (D'Silva and D'Souza, 1992a; Das *et al.*, 1996; Keshavachandran, 1996), shoot nodal explants from glass house raised plants (Boggetti *et al.*, 1999), embryo axis (Ananthakrishnan *et al.*, 1999), shoot tip and node/axillary buds (Keshavachandran and Shelja, 1999; Thimmappaiah and Shirly, 1999; Keshavachandran, 2004).

However, there has been very limited success in *in vitro* regeneration of cashew. For the successful use of tissue culture technology in cashew for crop improvement, an efficient and reproducible regeneration procedure is a pre-requisite. Reports on cashew indicate low rates of regeneration and abnormal morphogenetic responses (Jha, 1988; Hedge and Kulasekaran, 1994). Several factors such as tissue source and age and composition of medium influence regeneration frequency (Karp, 1991). Direct shoot bud regeneration was observed on cultures when proximal cotyledon halves were placed with their

petiolar end protruding from the medium. Similar observations were recorded in several plant species (Mehta and Ram, 1980; Rubos and Pryke, 1984; Mantell *et al*., 1998, Sharma and Bhojwani, 1990). Removal of the proximal region from cotyledonary explants led to the loss of the ability to develop adventitious shoots (Ananthakrishnan *et al*., 2002). Similar observations were also made on apple (Kouider *et al*., 1984; Rubos and Pryke, 1984). Philip (1984) also implies that in the material he used there is no limitation of regeneration to the proximal end of the cotyledon.

The present study was aimed at assessing and managing recalcitrance of *in vitro* cultures in cashew with respect to the phenolic exudation, endophytic microbial contamination, induction of rooting with *Agrobacterium rhizogenes* and improvement of transplantation success by treatment with AMF-PGPR consortium. Results obtained in the study on "Management of recalcitrancy in *in vitro* cultures of cashew (*Anacardium occidentale* L.)" are discussed in this chapter.

5.1 ISOLATION, IDENTIFICATION AND MANAGEMENT OF ENDOPHYTIC AND COVERT BACTERIA

5.1.1 Isolation of endophytic and covert bacteria

The present study confirms earlier reports on the possible survival of bacteria in endophytic or covert form in plant tissue cultures (Cassells, 1991; Viss *et al*., 1991; Leifert and Waites, 1992; Horsch and King, 1983; Kamoun *et al*., 1998) and demonstrates the essentiality of a sequential screening of cultures involving visual examination, indexing of medium followed by tissue-indexing to detect such bacteria. Three types of bacterial contamination / association were found to be prevalent in the cultures. This included obviously visible growth which could be easily picked up, endophytic bacteria detection which needed indexing of tissue and covert bacteria in the medium which might be brought out

through medium indexing. The last two types may go undetected and gradually spread to more cultures, contributing to culture degeneration (Thomas, 2004a). In the absence of indexing, bacteria harbouring in cultures would escape detection as they are not suspected as contaminated. Covert or inconspicuous bacteria *in vitro*, unless introduced intentionally, is highly undesirable as they can interfere with research conclusions and act as a potential threat to the micropropagation industry (Leifert and Woodward, 1998), *in vitro* gene banks (Houwe and Swennen, 2000) and safe exchange of germplasm (Salih *et al*., 2001).

Indexing of medium is preferably done 1-2 weeks prior to the intended date of sub-culturing, while tissue-indexing is best done at sub-culturing. In the present study, contaminants showed delayed growth that was seen on the bacteriological indexing medium (BIM) after one month. Splitting the stem facilitates direct contact of bacteria with the BIM. Distribution of endophytes may be uneven (Cassels, 2000) as observed in this study too and it is desirable to use representative tissue from as many different parts of plantlets as possible.

Bacterial contaminants in tissue cultures may be latent to the extent that they are normally visible on tissue culture medium but their effects on plant growth whether inhibitory, null or promotive are not clearly known (Thomas, 2004a).

5.1.2 Cultural characterization of endophytic and covert bacteria

The cultural characters of KAU-EC1, KAU-CC1 and KAU-CC2 were studied using nutrient agar media. KAU-EC1 gave rise to large mucoid, opaque, circular convex colonies on nutrient agar medium. KAU-CC 1 gave rise to circular, opaque yellow to creamy colonies. KAU-CC2 gave rise to circular, smooth, undulate, convex and yellow coloured colonies.

KAU-EC1, KAU-CC1 and KAU-CC2 appeared red after Gram staining reaction indicating Gram negative nature which was further confirmed by the KOH test. This reaction depends on the lysis of Gram negative cell in dilute alkali (3 per cent KOH) solution and as a result cellular DNA is released and the suspension turns viscous (Suslow *et al.*, 1982). For Gram negative bacteria, the cell wall is a very thin layer of peptidoglycan and they have an outer membrane composed of phospholipids, proteins, lipoproteins and lipopolysaccharides. In the Gram staining technique, crystal violet, which is the primary stain is bound by Gram's iodine (mordant) and an insoluble complex is formed. The decolourising agent, ethyl alcohol, washes off the complex in case of Gram negative bacterium. Safranin which is the counter stain gives the red colour.

5.1.3 MOLECULAR IDENTIFICATION OF ENDOPHYTIC AND COVERT BACTERIA

5.1.3.1 Isolation of bacterial Genomic DNA

Isolation of good quality of DNA is a pre-requisite for PCR. The genomic DNA isolation protocols reported by Schleif and Wensink (1981) was tested for isolation of bacterial genomic DNA of KAU-ECI, KAU-CC1 and KAU-CC2. The bacterial isolates were cultured overnight in NA broth with vigorous shaking before isolating DNA. The DNA isolated by this method was analyzed by agarose gel electrophoresis using a 0.8 per cent agarose gel. Good quality DNA was indicated by discrete bands. The quantity of DNA obtained by the Schleif and Wensink protocol was estimated by NanoDrop® ND-1000 spectrophotometer. The quantity of DNA isolated from 3 ml of 24 hr old culture of KAU-ECI, KAU-CC1 and KAU-CC2 and the ratio of absorbance at 260 nm to absorbance at 280 nm ranged from Table 8. The value of A_{260}/A_{280} between 1.77 and 1.85 indicates relatively pure DNA.

Cell walls of bacteria have been removed by lysozyme treatment. EDTA is one of the reagents used in DNA isolation. EDTA protects the DNA from the action of DNase enzyme by chelating and blocking the action of Mg^{2+} ions, which are the major cofactors of DNase enzyme.

The detergent used was SDS which acts as a nuclease inhibitor and also dissolves membranes. Efficient extraction of cell extracts or solution containing nucleic acids are most often performed with a series of phenol and phenol:chloroform extractions. Both phenol and chloroform denature proteins which get solubilized in organic phase or interphase, while nucleic acids remain in the aqueous phase. Chloroform is mixed with phenol to increase the efficiency which is due to the ability of chloroform to denature proteins. It helps in removal of lipids, thus improving separation of nucleic acids into the aqueous phase. To reduce the foaming caused by chloroform, isoamyl alcohol is usually added. Chloroform:isoamyl alcohol improves deproteinization.

Ethanol precipitation can concentrate DNA and purify the DNA after the phenol extractions. Basically, combining the DNA sample with salt and ethanol, at -20°C or lower, precipitates the DNA. The precipitated salt of the nucleic acid is then sedimented by centrifugation, the ethanol supernatant is removed, and the DNA pellet is resuspended in a buffer.

5.1.3.2 Amplification of 16S rDNA by PCR

Polymerase chain reaction was performed for the amplification of 16S rDNA gene. 16S rRNA gene has received more attention than 5S or 23S rRNA genes as it is more appropriate sizewise for study. The 16S rRNA molecules contain both highly conserved regions and variable regions (Stackebrandt *et al*., 1991). The highly conserved regions provide priming sites suitable for the polymerase chain reaction and sequencing applications. This molecular method was successfully used for compositional analysis of natural

microbial communities in many different environments (Weisburg *et al*., 1991; Hugenholtz *et al*., 1998; 2001; Dojka *et al*., 2000; Ravenschlag *et al*., 2000; Reysenbach *et al*., 2000; Derakshani *et al*., 2001; Hashidoko *et al*., 2002; Stein *et al*., 2002). 16S rDNA gene was amplified using two universal bacterial primers: $16S_{43\text{-}63}$ and $16S_{1404\text{-}1387}$. The use of $16S_{43\text{-}63}$ and $16S_{1404\text{-}1387}$ primers have been reported (Radeva and Selenska-Pobell, 2005). The PCR product when checked on agarose gel indicated the presence of band 1.3 kb. The band was later eluted and cloned in pGEM-T vector. By using the 16S primers a single amplification product having molecular size of about 1.3 kb was obtained for KAU-EC1, KAU-CC1 and KAU-CC2.

5.1.3.3 Transformation

Amplified DNA fragments of KAU-EC1, KAU-CC1 and KAU-CC2 were ligated into pGEM-T vector and transformed into *E. coli*. JM 109 cells. Competence of JM 109 *E. coli* cells was confirmed by transforming the cells with plasmid (pUC 18) having ampicillin resistance. *E. coli* cells alone could not grow on ampicillin containing media, as they lack the gene for ampicillin resistance. But the competent cells harbouring the plasmid could grow in the media. In the present study, large number of blue colonies (Plate 31 A) was observed on LB/ampicillin plate after overnight incubation at 37°C, confirming the competence of *E. coli* (JM 109) cells for transformation.

For cloning of 16S rDNA gene, pGEM-T vector of approximately 3.1 Kbp size was used. It contains T7 and SP6 DNA polymerase promoters that flank a multiple cloning region within the α peptide-coding region of the enzyme β-galactosidase. Thus, due to insertional inactivation of the α peptide region, the recombinants can be directly identified by the blue-white screening of indicator plates. The vector contains multiple restriction sites within the multiple cloning regions thus facilitating easy ligation of insert and its release by digestion with the restriction enzyme.

In the present study, the ligated product containing 16S rDNA gene was used to transform the competent cells. The white colonies could be easily distinguished and picked up from the selection media containing 5-bromo-4-chloro-3-indolyl β-D galactoside (X-gal) and isopropyl thiogalactoside (IPTG).

pGEMT vector contained polycloning sites inside a gene encoding for β-galactosidase. Thus, insertion of a new sequence would disrupt the reading frame of galactosidase encoding gene. As a result of α-complementation, the bacterial cell and vector together provided the complete protein, because one part of the gene was present in the bacteria and the other in the vector (Ullmann *et al.*, 1967). The colonies which have not taken up the plasmid further utilized the substrate and appeared as blue colonies on chromogenetic substrate, X gal (Horwitz *et al.*, 1964). Due to the disruption of α-complementation, all the transformed colonies harbouring the recombinant plasmid appeared as white (Plate 15 B).

The plasmid DNA isolated from white and blue colonies gave bands with different molecular weights. All the plasmids from white colonies had higher molecular weight than the plasmid of blue colonies due to the presence of the insert.

The presence of the insert was further confirmed by PCR amplification of the plasmids with M13 primers. Amplification was present in the case of white colonies. There was no amplification in blue colonies.

5.1.3.4 Sequencing of clones

Since there is no automated sequencing facility at KAU and it would be highly laborious to sequence the clones manually, the autosourcing facility providing by Bangalore Genei, was utilized.

5.1.3.5 *In silico* analysis of sequences

The sequences obtained after cloning, were subjected to vector screening to delete the sequences of vector if any present. Vector screening of KAU-EC1 showed significant similarity with vector from the region starting from 10-47 base pairs. Vector screen of KAU-CC1 showed strong match to vector starting from 10-48 bp. Vector screen of KAU-CC2 showed strong match to vector from 9-46 bp. Hence these regions showing similarity to vector were deleted and only the sequences of gene were retrieved.

Homology search through BLAST is a heuristic method to find the highest scoring locally optimal alignments between a query sequence and a database sequence (Altschul *et al.*, 1997). The BLAST analysis can determine the sequence homology to predict the identity and function of the query sequence. When the cloned sequences of KAU-EC1 was subjected to Blastn homology search, it was identified as *Klebsiella pneumoniae* strain SA-D6-7. Sequence of KAU-EC1 showed 99 per cent homology with *Klebsiella pneumoniae* strain SA-D6-7 16S ribosomal RNA gene present in NCBI data bank (Wang and Dang, 2008).

When the cloned sequences of KAU-CC1 was subjected to Blastn homology search, it was identified as *Pantoea agglomerans* strain XW123. Sequence of KAU-CC1 showed 98 per cent homology with *Pantoea agglomerans* strain XW123 16S ribosomal RNA gene present in NCBI data bank (Lin *et al.*, 2005), sequence of KAU-CC2 showed 95 per cent homology with *Stenotrophomonas maltophila* strain H2S8 16S ribosomal RNA gene (Chopade *et al.*, 2007). Therefore KAU-CC2 was identified as *Stenotrophomonas maltophila* strain H2S8.

5.1.3.6 Management of endophytic and covert bacteria

Endophytic bacteria (KAU-EC1) was resistant to six antibiotics tested. Covert bacteria (KAU-CC1) managed by adding 50 mg l^{-1}cefotaxime to media. Covert bacteria (KAU-CC2) was resistant to six antibiotics tested.

5.2 ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI FROM NODAL SEGMENTS COLLECTED FROM FIELD PLANTS

Three fungal species were detected from 2, 4 and 6 week after culturing of nodal segments derived from field plants. Fungal species were purified by inoculating into PDA media and sent to the National Centre of Fungal Taxonomy, New Delhi for identification of fungi. The fungal species were identified as *Fusarium oxysporum*, *Fusarium moniliformae* and *Botryodiplodia theobromae*.

5.2.1 Management of endophytic fungi

Spraying with 0.1 per cent Bavistin onto field explants and treatment with 0.1% Bavistin for 15 min. before surface sterilization of explants were effective to reduce the contamination. Maximum survival per cent (45 %) obtained during April. $CuSO_4$ at 100, 150 and 200 mg l^{-1} was added into establishment media. Bavistin at 0.025, 0.05 and 0.1% was also added into different media. But addition of Bavistin and $CuSO_4$ was less effective to reduce contamination.

5.3 ESTIMATION OF TOTAL PHENOL

Preece and Compton (1991) reported that browning of explants and media due to exudation of phenolics is one of the serious bottle-necks in the establishment of cultures from trees. It is found to be extremely severe in cashew

as it contains high amounts of phenols (D'Silva and D'Souza, 1993). In the present study treatment with 0.2 per cent PVP, 100 mgl^{-1} ascorbic acid and 0.5 per cent ascorbic acid was carried out. Friedman two way analysis of variance of ranks was carried out to detect the differential leaching of phenol into liquid media and sterile water. The Friedman test statistic of phenol leached into liquid media was 5.16 at 15.1 per cent level of significance. The Friedman test statistic of phenol leached into sterile water was 14.04 at 0.3 per cent level of significance. The highest mean rank score of phenol leached into liquid media was obtained for 0.2 per cent PVP followed by 0.5 per cent activated charcoal, followed by 100 mg ascorbic acid and control. Thus 0.2 per cent PVP was very effective to adsorb phenols. Treatment with ascorbic acid was not that effective as compared to PVP treatment.

Comparative evaluation of phenol leached out into liquid media based on constitutive relative increase was done for four treatments (Table 28). Treatment with 0.2 per cent showed that trivial decrease was noticed during 15-17.5 hrs time interval and steady increase was noticed during 17.5-20 hrs time interval and constitutive increase was noticed during 20-48 hrs time interval. Treatment with 100 mg ascorbic acid showed that steady increase was noticed during 15-17.5 hrs time interval and constitutive decrease was noticed during successive time intervals. Treatment with 0.5 per cent activated charcoal showed that trivial decrease was noticed during 17.5-20 hrs time interval and constitutive increase was noticed during 20-48 hrs and 48-53 hrs time interval.

Comparative evaluation of phenol leached out into sterile water based on constitutive relative increase was done for four treatments (Table 29). Relative increase of treatment with 0.2 per cent estimated null increase during 15-17.5 hrs and 17.5-20 hrs time interval and steady increase was noticed during 20-48 hrs time interval. Relative increase of treatment with 100 mg ascorbic acid showed that null increase was noticed during 15-17.5 hrs time interval and steady decrease was noticed during successive time intervals. Relative increase of

Table 29. Comparative evaluation of phenol leached out into sterile water based on constitutive relative increase

Time interval	Control	0.2%PVP	100 mg ascorbic acid	0.5% activated charcoal
15-17.5 hr	(26.83)	(0)	(0)	(1.64)
17.5-20 hr	(13.46)	(0)	(-11.29)	(-9.09)
20-48 hr	(-22.91)	(12.69)	(-9.09)	(-3.45)

Table 30. Comparative evaluation of phenol leached out into liquid media based on constitutive relative increase

Time interval	Control	0.2%PVP	100 mg ascorbic acid	0.5% activated charcoal
15-17.5 hr	(-5.405)	(-1.66)	(3.03)	(6.15)
17.5-20 hr	(-10.23)	(4.37)	(14.71)	(-0.35)
20-48 hr	(5.01)	(28.57)	(-25.64)	(51.11)
48-53 hr	(20)	(8.15)	(-12.41)	(8.82)

treatment with 0.5 per cent activated charcoal showed that trivial increase was noticed during 15-17.5 hrs time interval and steady decrease was noticed during 17.5-20 hrs and 20-48 hrs time interval.

5.4 STANDARDIZATION OF *IN VITRO* REGENERATION

5.4.1 EXPLANTS FROM FIELD PLANT

5.4.1.1 Standardization of surface sterilization

In the present study maximum survival per cent (90) was observed with treatment with 0.1 per cent $HgCl_2$ for 2 minutes. Thimmappaiah *et al*. (2002) reported that surface sterilization of explants with $HgCl_2$ for 5-8 min. was effective. Survival percentage was very low at 5 min., the treatment resulting browning and death of explants.

5.4.1.2 Effect of basal media on regeneration

Two basal media (full MS and half MS) tested for culture establishment showed that MS with half strength nutrients was ideal for establishment. MS medium with half major nutrients and 0.4 g l^{-1} glutamine had a significant effect on shoot growth. Similarly, Mantell *et al.* (1998) and Boggetti *et al*. (1999) found MS medium with half strength major nutrients ideal for culture establishment.

5.4.1.3 Effect of season on survival and bud break of nodal cultures

The culture establishment from young trees was affected by the prevalent conditions of high humidity and adverse weather conditions in this region. Season influenced contamination and bud break. Contamination was high during the rainy season (June to October) due to incessant rains and high humidity

and low during dry months (March to May) due to cessation of rains and reduced humidity. On the other hand, bud break was low during the rainy season due to dormancy of shoots and inclement weather and was high during dry months mainly on account of warm weather.

Thimmappaiah *et al*. (2002) reported that establishment of shoot cultures was affected by season of collection, source and type of explant.

5.4.1.4 Culture establishment

A maximum of forty five per cent bud break was obtained on ½ MS + 0.4 g l^{-1} glutamine + 0.2% PVP + 2.25 g l^{-1} phytagel. This result is in conformity with the results obtained by Thimmappaiah *et al*. (2002) who reported that fifty five per cent bud break in 1-2 year old cashew grafts with the combination ½ MS + 0.4 g l^{-1} glutamine + 0.1% PVP + 2.25 g l^{-1} phytogel. Thimmappaiah *et al.* (2002) reported that inclusion of L-glutamine enhanced survival rate (40%) and bud break (30%) then the control where survival rate and bud break were 36 and 13.3 per cent, respectively. L-glutamine is a organic source of nitrogen found to reduce browning of cultured explants (Pierik, 1990).

5.4.1.5 Multiplication and elongation

Thidiazuron is known to be effective for multiple shoot induction in woody plants. Such a multiple shoot induction was reported in nodal explants derived from a 10 year old cashew tree by Thimmappaiah *et al.* (2002) and nodal explants of mature cashew tree showed two to six multiple buds per culture. In the present study, two to three multiple buds per culture were observed. Transfer of multiple shoots onto hormone free medium was necessary with the shoots obtained from the TDZ containing medium for better performance. Maximum elongation of 1 cm was observed in shoot bud.

5.4.1.6 Rooting

No rooting could be observed with the combination ½ MS + 1 mg l^{-1} IBA + 1 mg l^{-1} IAA. Shoots were inoculated into the media after pulse treatment with IBA 24 mg l^{-1} for 24 hour and also pulse treatment with NAA. This is in accordance with the results of Thimmappaiah *et al.* (2002) who reported that rooting of long microshoots attempted both by pulse treatment (10 mM NAA) and *in vitro* rooting on half MS medium containing different levels of NAA, IBA and their combinations failed to induce any root.

5.4.2 *In vitro* seed germination

The seed germination was favoured in MS medium supplemented with 3.5 mg l^{-1} kinetin + 1 mg l^{-1} NAA, 3 per cent sucrose, 0.05 per cent activated charcoal. Eighty per cent of the seeds germinated within 15 days where as only twenty five per cent seeds germinated within 30 days under 16 hours (1000 lux) photoperiod. Keshavachandran (2004) reported seed germination was favoured in solid MS medium supplemented with charcoal, 3 per cent sucrose, kinetin, NAA and brassinolide.

MS medium was shown previously to be effective in enhancing frequency of regeneration in cashew tissue culture (Keshavachandran and Khader, 1990; D'Silva and D'Souza, 1992a; Das *et al.*, 1996; Boggetti *et al.*, 1999; Keshavachandran, 2004). Thimmappaiah *et al.* (2001) obtained 98.4 per cent germination in medium containing WPM salts with NAA. Philip (1984) reported organogenesis from cotyledonary explants of cashew on LS medium (Lin and Staba, 1961) supplemented with kinetin and IAA. In the present study, kinetin at 3.5 mg l^{-1} was the most effective cytokinin in inducing shoots from proximal end of cotyledon with NAA.

5.4.3 EXPLANTS FROM *IN VITRO* SEEDLINGS

5.4.3.1 Culture establishment and multiplication

Hundred per cent regeneration response were obtained from nodal segments, cotyledonary nodes and shoot tips in all the concentrations of BA and IAA. Application of cytokinin at relatively high doses has been recommended for *in vitro* cashew bud development in microshoots and cotyledonary nodes (Lievens *et al*., 1989; Leva and Falcone, 1990; D'Silva and D'Souza, 1992a; Das *et al*., 1996). From both shoot tips and nodal segments, the combination MS + 1.5 mg l^{-1} BA + 1 mg l^{-1} IAA produced maximum number of shoot buds. On taking maximum number of shoot buds and shoot elongation together, maximum multiplication was obtained from MS with 2.5 mg l^{-1} BA and 1 mg l^{-1} IAA.

Lakshmi Sita (1989) reported 10-13 cm shoot initials with half cm length in a medium with BA (0.5 mg l^{-1}) and 3 per cent sucrose. Keshavachandran and Khader (1990) obtained 3-5 shoot buds/cotyledonary nodes on SH medium supplemented with 5-10 mg l^{-1} BA and 0.5 mg l^{-1} IAA.

D'Silva and D'Souza (1992a, b) found that multiple shoots were induced in cotyledonary nodes cultured on MS medium supplement with 4 per cent sucrose, 5.3 gl^{-1} maltose and 5 mg l^{-1} BA.

Das *et al*. (1996) observed that cotyledonary nodes produced 12 buds than other explants on MS medium containing 1 mg l^{-1} BA, 0.5 mg l^{-1} kinetin and 2.0 mg l^{-1} zeatin.

Thimmappaiah (1997) reported multiple shoot induction on MS medium supplemented with 2.25 mg l^{-1} BA and 0.2 mg l^{-1} IBA. In the present study 2.2 shoot buds/cotyledonary segments with 2 cm length and 2.2 shoot buds/nodal segments with 3 cm length was observed on MS with 2.5 mg l^{-1} BA

and 1 mg l^{-1} IAA. Similarly 3.4 shoot buds/cotyledonary segments with 1.2 cm length and 2.3 shoot buds/nodal segments with 1.4 cm length was observed on MS with 1.5 mg l^{-1} BA and 1 mg l^{-1} IAA.

5.4.3.2 Elongation

Maximum elongation was obtained on MS with 2 mg l^{-1} BA and 400 mg l^{-1} glutamic acid. Boggetti *et al* (1999) reported that continuous presence of high concentration of cytokinin suppresses bud sprouting and inhibited shoot elongation. Therefore, BA at low concentration was found to be optimal for shoot proliferation and shoot elongation.

5.4.3.3 Rooting

Rooting in half MS with 1 mg l^{-1} IBA and 1 mg l^{-1} IAA after pulse treatment with IBA 24 mg l^{-1} for 24 hrs was found to be highly effective for inducing roots *in vitro*. Eighty per cent of rooting was obtained in this medium.

The two-step procedure proved effective for rooting of cashew microshoots. A two-step rooting procedure has also been reported in cashew (Das *et al*., 1996). Das *et al*. (1996) obtained 40 per cent rooting by exposing cashew microshoots to 245 µm IBA for 2 hr. Ananthakrishnan *et al*. (2002) reported 55 per cent rooting in 122.6 µm IBA for 72 hrs. Shoots were transferred to half-strength MS solid medium containing IBA (4.9 µm) and IAA (5.7 µm).

Keshavachandran and Riji (2005) reported *in vitro* rooting with pulse treatment with IBA for 24 hours followed by transfer to ½ MS liquid medium supplemented with low levels of IBA and 200 mg l^{-1} glutamic acid.

In the present study, eighty per cent rooting was observed compared to results of Das *et al*. (1996) and Ananthakrishnan *et al*. (2002). The

lower auxin concentration used for rooting improves plant quality by not causing callus formation.

The effectiveness of IBA in rooting has been reported in many species. According to Ludvig-Muller (2000) transport velocity of IBA was markedly slower compared to that of IAA and NAA. The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus its better function in inducing roots (Nickell, 1982).

5.4.3.3.1 Effect of *Glomus fasciculatum* on *in vitro* rooting

The procedure reported by Gerdemann and Nicolson (1963) was used for the isolatoion of AM fungal spores from the rhizosphere soil. Spores after surface sterilization were inoculated into solid media before the initiation of rooting and after the initiation of rooting. Per cent root colonization was assessed by using the method described by Phillips and Hayman (1970). In the present study, there was no colonization for 30 days after inoculation of *Glomus fasciculatum* spores into the media.

5.4.3.3.2 Effect of *Bacillus subtilis* on *in vitro* rooting

Twenty four hrs old *Bacillus subtilis* culture was used as inoculum. Bacterial inoculum (10^8 cfu) was added into the media at two stages (1) after the initiation of roots (2) three days before the transplanting. In the present study there is no positive influence of *Bacillus subtilis* on *in vitro* rooting, because the basal portion of explants decayed after one week, due to higher multiplication rate of the bacteria.

5.4.3.3.3 ROOT INDUCTION WITH *AGROBACTERIUM RHIZOGENES*

5.4.3.3.3.1 Culturing and sensitivity screening of *Agrobacterium* and explants

Complete elimination of the bacteria from the explants after co-cultivation is very essential; otherwise it will interfere with the growth and root production of explants. Overgrowth of the bacteria causes death of the explants and disrupts the experiment. Elimination of the bacteria from the explants is done using antibiotics. The antibiotic chosen should be such that it efficiently kills the bacteria; at the same time it does not affect the growth and morphogenesis of the explants. The most commonly used antibiotics for this purpose were cefotaxime, ampicillin and carbencillin. However the sensitivity of each *A. rhizogenes* strain and explants towards these antibiotics has to be studied carefully so as to find out the most appropriate one to be used in the further experiments.

Both the strains of *A. rhizogenes* used (MTCC 2364 & MTCC 532) were found to be sensitive to cefotaxime, whereas, the strain MTCC 2364 showed resistance to ampicillin and carbencillin. The strain MTCC 532 also showed resistance to ampicillin. Cefotaxime at 500 mg l^{-1} killed the two strains of *A. rhizogenes*. So 500 mg l^{-1} cefotaxime was taken as the optimum concentration of antibiotics to kill *A. rhizogenes* strains under study. All the explants used in the transformation event were found to be healthy at this concentration (500 mg l^{-1}) of cefotaxime. Similarly in many reports, 500 mg l^{-1} cefotaxime was selected as the antibiotic concentration to kill *A. rhizogenes* strains (Koike *et al.*, 2003; Zdravkovic-Korac *et al.*, 2004).

5.4.3.3.3.2 Preculturing of explants

Elongated shoots were used as the explant. The explants were cultured for two days on ½ MS medium without antibiotics prior to transformation. This was done to make the explants acquainted to the new culture condition, since each

explant was now in direct contact with the media. The cell division will be initiated and the endogenous hormones will be used up during this phase, thereby preparing the explants for transformation.

5.4.3.3.3.3 Wounding of explants

A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds, such as acetosyringone, that activate the vir region of the bacterial plasmid (Binns and Thomashow, 1988).

In the present study, a small cut was given at the basal portion and explants were immersed in 0.5 ml of bacterial suspension contained in small test tubes. Roots were initiated at the surface or near the surface of the basal cut without visible callus proliferation. This is in accordance with the observations of Damiano *et al.* (1995) who reported that *in vitro* rooting was obtained in almond cultivar super through the infection of *Agrobacterium rhizogenes* w.t. strain 1855 NCPPB, at the base of the microcuttings.

Elliot (1951) reported that *A. rhizogenes* induces proliferation of roots at the site of infection. This is due to the transfer to the host cells of a portion of Ri plasmids (Root inducing) T-DNA (Chilton *et al.*, 1982; White *et al.*, 1982; Willmitzer *et al.*, 1982). Also, root transformation using the Ri plasmid may result in a more effective root system (Torrey, 1988).

Wounding of explanted *Pinus nigra* primary explants followed by infection with *Agrobacterium rhizogenes* wild strains 8196, 153834 or with the pRi A4abc transcojungant strain of *A. tumefaciens* (C58 chromosomal background) resulted in adventious root induction (Mihaljevic *et al.*, 1999). Nilsson and Olsson (1997) hypothesized that only cells containing high levels of auxin and sucrose (which regulate *rol B* and *rol* C promoters, respectively) are

able to act as root meristem initials and they are also ideal targets for *A. rhizogenes* infection. Since ray cells and phloem cells are positioned in the region with the highest amount of sucrose and considerable amount of IAA, they could be convenient targets for *A. rhizogenes* infection. Therefore wounding of explants of *Anacardium occidentale* is favoured for inducing roots.

5.4.3.3.3.4 STANDARDIZATION OF ROOT INDUCTION

5.4.3.3.3.4.1 Influence of bacterial inoculum

The bacterial inoculum used affects the transformation frequencies. In the present study, when the bacterial suspension was used as the inoculum transformation was produced only by MTCC 2364 strain whereas no transformation was shown by MTCC 532.

Hawes *et al*. (1988) have reported that the motile strains of *Agrobacterium* exhibited virulence only in liquid medium but mutant strains (non motile) exhibit virulence when inoculated directly on wounds.

Strobel and Nachmias (1985) have observed increased root numbers and root mass in almond trees that had been treated with suspensions of *A. rhizogenes*. In accordance to the present results Das *et al*. (1996) reported cashew shoots formed roots after submerging the shoots in suspension of *A. rhizogenes*. The reports of successful root induction by using bacterial suspension were given by Damiano *et al*., 1995; Mihaljevic *et al*., 1996; Li and Leung, 2003. The strain MTCC 2364 was found to be virulent compared with MTCC 532.

5.4.3.3.3.4.2 Effect of co-culture period

Co-culture is an effective stable method for inducing the desired gene into plant DNA (Malabadi and Nataraja, 2003). Co-cultivation plays an

important role in the success of transformation. It is during this period that the vir genes are activated and the T-DNA is transferred into the plant cell. However, increasing the co-cultivation period might lead to necrosis and death of the explant due to the hypersensitive response of the tissue (Sarmento *et al.*, 1992). Hence the length of co-cultivation period should always be the shortest interval necessary to obtain maximum frequency of root induction with *Agrobacterium rhizogenes*.

In the present study, root induction was obtained only from shoot cultures under three days of co-culture period. After three days of co-culture period, there was overgrowth of bacteria which killed the explant tissues. Furthermore, after three days of co-cultivation it was difficult to eliminate the *Agrobacterium strains* completely. Similar results were reported by Pawlicki *et al.* (1992) in carrot.

In the present study, co-cultivation was carried in solid media. Damiano *et al.* (1995) reported that co-cultivation in liquid medium compared with solid seems to reduce the aggressivity of bacteria in producing the infection. Co-cultivation of basal meristem tissue with *Agrobacterium* offers the best approach for transformation of onions (Dommisse *et al.*, 1990).

Bacterial cells multiplied in the co-culture medium and after 24 hrs, the optimum quantity of bacteria were available for transformation and hence a higher transformation was achieved. After 36 hrs, the level of bacterial cells reached supra optimum and competitive inhibition of competent bacterial cells resulted in inhibition of transformation (Karmarkar *et al.*, 2001b).

Spano *et al.* (1988) reported that the T_L-DNA makes cells sensitive to auxin addressing them towards rhizogenic process. *Rol B* CORF ID was also found to be among the 18 ORFs present in T_L-DNA, the genetic determinant responsible for rooting (Cardarelli *et al.*, 1987; Estruch *et al.*, 1991) showing that

this gene codes for a β-glucosidase activity able to deconjugate active auxin from a biologically inactive IAA-A-glucoside. They found that that the level of free and active auxin in the plant cell depends on the amount of conjugates which is supposed to be genotypic specific. However, a substantial IAA homeostasis has been recently demonstrated in both *rol B* transgenic and control plants (Nilsson and Olsson, 1997). Damiano *et al.* (1995) reported that the rooting process seems to be precisely regulated in terms of hormonal balance and hormone sensitivity. He observed root induction in almond after a co-culture period of three days.

5.4.3.3.3.4.3 Effect of acetosyringone

Acetosyringone and related compounds are known to induce the expression of *Agrobacterium* virulence genes (Stachel *et al.*, 1986; Bolton *et al.*, 1986; Binns and Thomashow, 1988) and the resulting circulation of T-DNA (Usami *et al.*, 1987).

In the present study, acetosyringone dissolved in DMSO (Dimethyl sulphoxide) was supplemented at a concentration of 10 mM, 20 mM prior to (bacterial suspension) or/and during co-cultivation in MS media in order to increase the efficiency of *Agrobacterium*. Tepfer (1984) reported that successful infection of some species could be achieved by the addition of acetosyringone. Venkatachalam *et al.* (2007) reported that 20 mM acetosyringone influences transformation in rubber. In the present study, no positive influence was noticed with MTCC 2364.

It appears that the phenolic compounds released by the plant cells were sufficient to induce the virulence genes and stimulate the genetic transformation. This is in agreement with the results of Pawlicki (1992) with carrot, Sangwan *et al.* (1991) on Datura and Kumar *et al.* (2002) on lucerne by using *A. tumefaciens* strains. The strain MTCC 532 failed to produce root

induction with *Agrobacterium rhizogenes* both in presence and absence of acetosyringone.

5.4.3.3.3.4.4 Standaridization of explants for rooting with *Agrobacterium rhizogenes*

It has been reported that the virulence of *Agrobacterium* strains varies among the plant hosts (Hobbs *et al*., 1989) and that the transformation efficiency of host species can vary between different bacterial strains (Godwin *et al*., 1991).

In the present study, different explants such as shoot cultures and nodal segments of *Anacardium occidentale* were used. Nodal segments failed to produce root induction with the strain MTCC 2364 and MTCC 532. The shoot cultures of *Anacardium occidentale* produced roots with the strain MTCC 2364.

Stachel *et al*. (1986) reported that the induction of vir genes was not merely a response of *Agrobacterium* to necrotic plant cells, but it required an active plant cellular metabolism. Citovsky *et al*. (1991) stated that cell division and DNA synthesis are involved in the incorporation of T-DNA into the plant genome. Therefore absence of cell division may prevent successful T-DNA transfer. The explant cells differ in their DNA synthesis and cell division ability due to the difference in the physiological maturity of the cells (Karmarkar, 2001b).

Nilsson and Olsson (1997) hypothesized that only cells containing high levels of auxins and sucrose (which regulate *rol* B and *rol* C promoters respectively) are able to act as root meristem initials and are also ideal targets for *A. rhizogenes* infection.

Potrykus (1990) did a critical assessment of the *Agrobacterium* mediated gene transfer process. He stated that the most important factor for successful transformation by *Agrobacterium* was the wound response and the plant tissues differ in their wound response. He further stated that the explants with a pronounced wound response develop larger populations of wounds adjascent competent cells for regeneration and transformation. He suggested that the explants or plant species recalcitrant to transformation with *Agrobacterium* probably do not express appropriate wound response.

It is possible that the shoot cultures inoculated with the bacterium were more sensitive to increased auxin supply since *A. rhizogenes* is known to encode genes that increase auxin sensitivity to plant tissue (McAfee *et al.*, 1993; Hatta *et al.*, 1996). It can therefore be attributed that wound sites associated with actively dividing cells of shoot cultures show root induction with *A. rhizogenes*. The TL-DNA (rol B in particular) confers the competence to respond to auxins (Cardarelli *et al.*, 1987). Therefore explants capable of auxin synthesis (shoot cultures) showed root induction with *A. rhizogenes* since these cells contains high levels of auxins and sucrose that can act as root meristem initials which are the ideal targets of *Agrobacterium* infection. McAfee *et al.* (1993) reported that the bacterium may be contributing to the rooting process in *Pinus* sp. and *Larch* sp. by modifying the root environment by means of hormonal or other secretions or by low copy transformation of some cells. Strobel *et al.* (1988), working with olive trees, found that T-DNA is present in such low copy numbers that the demonstration of transformation in roots using the Southern blotting technique with labelled T-DNA as a probe is very difficult. After opine and molecular analysis for TR- and TL-DNA, Bassil *et al.* (1991) were unable to determine if the roots induced on hazelnut cuttings contained transformed tissues. Improved root growth when *Pinus sylvestris* was co-cultivated with *Agrobacterium* spp. has also been attributed the increased amount of organic acids (lactic, fumaric, citric) released in the rhizosphere (Leyval and Berthelin, 1989). Wordragon *et al.* (1992) using an *A. rhizogenes* transconjugent strain containing the GUSintron reporter

concluded that root induction in chrysanthemum was the result of transient expression of auxin synthase genes on the TR-DNA, not the result of stable gene transfer.

5.4.3.3.3.4.5 Efficiency of strains in inducing root induction with *Agrobacterium rhizogenes*

Significant difference was observed between the root induction of different strains of *Agrobacterium*. In the present study MTCC 2364 showed root induction in *Anacardium oaccidentale*. The strain MTCC 532 however failed to produce any successful transformation.

Petit *et al*. (1986) studied the pathogenicity of different strains of *A. rhizogenes*. They suggested that the difference in host range and pathogenicity of *A. rhizogenes* strains is due to the difference in the plasmids they harbour. Similarly Cardarelli *et al*. (1987) attributed the difference in transformation ability of different strains of *Agrobacterium* to the plasmids harboured by them.

Strain MTCC 2364 and strain MTCC532 are agropine type Ri plasmids. Sevon and Oksman-Caldentey (2002) reported that agropine-type Ri plasmids are considered to be the most virulent and therefore more often used for *Agrobacterium* mediated genetic transfer.

Rhodes *et al*. (1989) reported that the agropine strains (e.g. 15834) have wide host range that is attributed to the presence of TR-DNA fragment of the T-DNA harbouring genes for auxin synthesis (tms 1 and tms 2). These genes trigger cellular division by auxin synthesis due to which these strains are able to transform a wide range of species. On the contrary, the mannopine strains and cucumopine strains are deficit in the TR-DNA fragment; hence they cannot trigger auxin synthesis and so can infect only a limited number of hosts.

McAfee *et al.* (1993) and Hatta *et al.* (1996) also found that different strains of *A. rhizogenes* brought about different rooting responses in pine (*Pinus*) and larch (*Larix*) spp. and in jujube (*Ziziphus jujube*) respectively.

5.4.3.3.3.4.6 Number of days for root induction with *A. rhizogenes*

Root induction with *Agrobacterum rhizogenes* was achieved in a time period of 4-8 weeks in the majority of the plant species. In the present study 25-30 days was taken for root induction with *A. rhizogenes* from shoot cultures. This is in accordance with the result of Das *et al.* (1996) who reported that rooting of shoots of cashew with *Agrobacterium rhizogenes* was achieved within a time period of 21-30 days.

The roots developed 10 days after infection in almond (*Prunus dulcis*) (Damiano *et al.*, 1995), 3 weeks after infection in *Allium* sp. (Dommisse *et al.*, 1990), 4-6 weeks after infection in *Pinus radiate* (Li and Leung, 2003) 8 weeks in *Pinus banksiana* and *Larix laricina* (McAfeee *et al.*, 1993), 8-6 weeks after infection in *Pinus nigra* (Mihaljevic *et al.*, 1996).

McAfee *et al.* (1993) did not detect T-DNA in the induced roots of *Pinus monticola, P. banksiana* and *Larix laricina* using the left T-DNA region of pRiA4b as DNA probe. The authors suggested that no T-DNA transfer had occurred or that it was present at a lower level than that detectable by the Southern blot procedure. Bassil *et al.* (1991) were unable to determine if the roots induced on hazel nut cuttings contained transformed tissue after opine and molecular analysis for TR and TL-DNA. In these cases, root induction was probably due to an improvement of rooting environment by the bacterium (McAfee *et al.*, 1993; Simpson *et al.*, 1986). Falasca *et al.* (2000) conducted an experiment to investigate how *A. rhizogenes* triggers *de novo* root formation in a recalcitrant walnut plant. They found that rooting on the infected cuttings was enhanced by IBA, which accelerated and increased root meristemoid formation, in

comparison with the treatment without hormone. PCR and Southern blotting analyses showed that root meristemoid formation was not accompanied by genetic transformation.

5.5 HARDENING AND PLANTING OUT

The rooted plants after exposure to light for one week were transferred to pots containing sterile sand (Nair and Mohanakumaran, 1993; Keshavachandran and Riji, 2005).

In the present study, four treatments were carried out during hardening stage. Among these four treatments, treatment with *Glomus fasciculatum* and *Bacillus subtilis* was observed with maximum survival per cent (70.85), which was followed by treatment with *Bacillus subtilis* alone.

5.5.1 EFFECT OF *GLOMUS FASCICULATUM* AND *BACILLUS SUBTILIS* ON SURVIVAL AND GROWTH OF MICROPROPAGATED CASHEW PLANTS

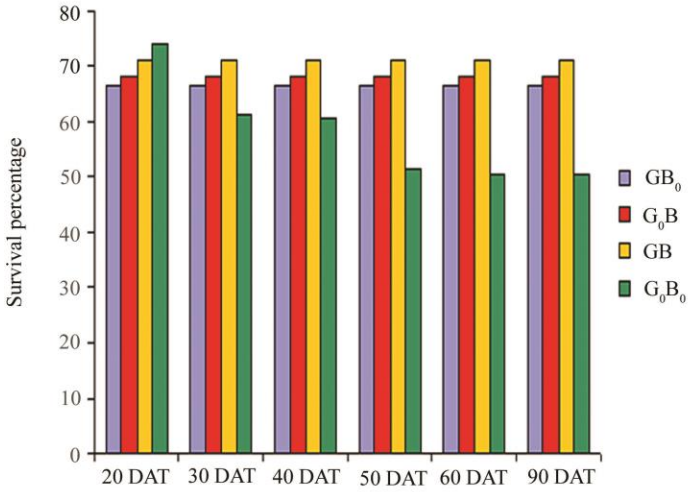
The beneficial organisms such as *Glomus fasciculatum, Bacillus subtilis* are well recognized for their positive influence on nutrition and health of crop plants. There are a large number of reports on exploiting the beneficial microorganisms for growth improvement and disease management of crop plants (Joseph, 1997; Aguilera-Gomez *et al.*, 1999; Sivaprasad *et al.*, 1999b; Pandey and Goswami, 2005). Strullu and Gerault (1977) and Strullu and Gourret (1980) reported that mycorrhiza formation on natural complexes of angiosperms and gymnosperms. Previous studies on micropropagated fruit trees have shown that inoculation with AM fungi at the time of transplanting the micropropagated plantlets from axenic to *ex vitro* conditions significantly improves their survival and vegetative growth of plants such as oil palm (Blal *et al.*, 1990), banana (Jaizme-Vega and Azcon, 1995; Lins *et al.*, 2003; Trindade *et al.*, 2003),

pineapple (Jaizme-Vega and Azcon, 1991, 1995; Guillemin *et al*., 1992; Varma and Schuepp, 1994), kiwi fruit (Schubert *et al*., 1990), apple (Uosukainen and Vesterg, 1994), pear and peach clonal rootstocks (Rapparini *et al*., 1994), peach and plum rootstocks (Sbrana *et al*., 1994; Fortuna *et al*., 1996 and 1998). Prunus rootstocks (Estaun *et al*., 1994; Berta *et al*., 1995; Estaun *et al*., 1999), avocado (Vidal *et al*. 1992; Azcon-Aguilar *et al*., 1992), olive (Marin-Zamora *et al*., 2002), persimmon (Marin *et al*., 2003).

5.5.1.1 Effect of *Glomus fasciculatum* and *Bacillus subtilis* on survival of tissue culture plantlets of cashew

The inoculation with mycorrhizal fungi and *Bacillus subtilis* either alone or in combination generally improved the survival rate of cashew plantlets during the hardening phase. Inoculation of *Glomus fasciculatum* and *Bacillus subtilis* resulted in 70.72 per cent survival of plantlets followed by *Bacillus subtilis* with 68.35 per cent survival and followed by *Glomus fasciculatum* with 66.68 per cent survival against 50.22 per cent recorded for control (Fig 9). In the present study, combination with *Glomus fasciculatum* and *Bacillus subtilis* showed a relatively higher survival rate than with mycorrizal fungi. The influence of mycorrhiza on survival, establishment and growth of tissue culture plantlets have been well documented in cassava (Calderan *et al*., 2001), sugarcane (Gosal *et al*., 2001), strawberry (Taylor and Harrier, 2001; Borrowska, 2002) and coffee (Fernandez *et al*., 2002). The high mortality rate of tissue culture plants are attributed to defective physiological features such as poor development of cuticle and epicuticular wax on newly emerging leaves (Leshem, 1983), impaired stomatal mechanism (Capellades *et al*., 1990), high transpiration rates (Preece and Sutter, 1991) and low photosynthetic rates (Lee *et al*., 1985). This leads to an uncontrolled water loss from the plant and consequent dehydration and wilting of the plant. Further, the poor development of roots and root hairs affects the uptake of water and nutrients (Reuther, 1986), because the defective vascular connection between root and shoot hinders the transport of water and nutrients (Sivaprasad

Fig. 8. Effect of *Glomus fasciculatum* and *Bacillus subtilis* on survival of tissue culture plantlets of cashew



and Sulochana, 2004) and mortality occurs due to desiccation. Again since the plants are raised under aseptic conditions and not exposed to any sort of microflora, the defence mechanism of the plant against the microbial infection is not triggered and hence, they are much vulnerable to microbial infection.

When mycorrhizal colonization takes place, the hyphae ramify inside the root cortical region and spread towards the soil and function synonymous to root hairs (Miller and Jastrow, 1990; Berta *et al.*, 1990; Schellenbaum *et al.*, 1991). The VAM hyphae attached to the root extend the absorption surface and directly translocate nutrients from the soil to the hyphae and arbuscules inside the root cortex where transfer to the plant occurs. Essentially this transfer involves carbohydrates from plant to fungus and minerals, especially phosphate, from fungus to plant and hence the deficiency of root hair formation of plantlets is corrected and proper absorption mechanism is provided to the plantlets (Berta *et al.*, 1990; Guillemin *et al.*, 1994; Thanuja *et al.*, 2002). The physiological changes due to AMF such as increased photosynthetic activity (Sivaprasad and Rai, 1985; Sanchez-Blanco *et al.*, 2004), enhanced phytohormone activity (Sivaprasad and Rai, 1987) favoured the early development of roots and fast growth and development of the plantlets. It is known that AMF induces systemic resistance against pathogens by triggering the production of PR proteins and phenolic compounds (Grandmaizon *et al.*, 1993; Sivaprasad *et al.*, 1995a; Gianinazzi-Pearson *et al.*, 1996). This trait of AMF makes the plant tolerant to pathogenic infection also.

Inoculation with *Glomus fasciculatum* alone showed 66.68 per cent survival. It is observed that 70.72 per cent survival recorded for both combination with *Glomus fasciculatum* and *Bacillus subtilis*. This indicates that the mortality occured before the establishment of mycorrhizal association on host system as it is known that mycorrhizal infection and subsequent development require around 20 days. Treatment with *Bacillus subtilis* alone resulted in 68.35 per cent survival. Hence, a combination of *Glomus fasciculatum* and *Bacillus subtilis* may give

better protection to the plantlets. In the uninoculated control by the 60th day of transplanting 49.78 per cent plants died and thereafter there was no mortality. This high mortality was due to wilting. In the later stage, the plantlets might have acclimatized with the outdoor conditions and hence there was no further mortality.

5.5.1.2 Effect of *Glomus fasciculatum* and *Bacillus subtilis* on height of tissue culture plantlets of cashew

Glomus fasciculatum and *Bacillus subtilis* significantly influences the height (cm) of tissue culture plantlets of cashew. The effect of plant height was evident from 30th day after transplanting on the 120th day after transplanting. The effect of *Glomus fasciculatum* and *Bacillus subtilis* was more pronounced with maximum plant height for GB followed by GBo. Among the treatments, treatment with *Glomus fasciculatum* and *Bacillus subtilis* was more pronounced followed by treatment with *Glomus fasciculatum* alone. Similarly *Bacillus* alone recorded significant increase in plant height over control.

5.5.1.3 Effect of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* on leaf formation in tissue culture plantlets of cashew

There was no significant difference between single inoculation or a combination of treatments over control. There was an increasing trend with various treatments from the 30th day after transplanting.

5.5.1.4 Effect of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* on root growth in tissue culture plantlets of cashew

The combination of inoculation of *Glomus fasciculatum* and *Bacillus subtilis* showed maximum root growth followed by treatment with *Glomus fasciculatum* alone. Similarly *Bacillus* alone recorded significant increase in root growth over control.

It is well documented that inoculation with *Glomus fasciculatum* improves the biomass production (Nagarajan *et al*., 1989; Kumari and Balasubramanian, 1993; Boucher *et al*., 1999; Mathur and Vyas, 1999) and growth characteristics such as plant height (Shashikara *et al*., 1999), leaf number (Aguilera-Gomez *et al*., 1999; Shashikara *et al*., 1999) and rooting (Anandaraj and Sarma, 1994a; Thanuja *et al*., 2002) of crop plants. The mycorrhiza induced improvement in plant growth characteristics is due to improved nutrient uptake (Harley and Smith, 1983; Joseph, 1997), water relations (Safir *et al*., 1971; Al-Karaki, 1998; Sanchez-Blanco *et al*., 2004) and disease tolerance (Sivaprasad *et al*., 1995a; Anandaraj *et al*., 1996; Robert, 1998; Kavitha, 2001; Thanuja *et al*, 2002). Esther and Mark (2007) reported that *Bacillus subtilis* used as biocontrol agent against *Fusarium solani* and *Pythium* in maize.

5.5.2 DETECTION OF AMF IN CASHEW ROOTS

5.5.2.1 Per cent root colonization

Per cent root colonization was assessed using the method described by Phillips and Hayman (1970). In the present study, the per cent root colonization 90 days after transplanting was 70 per cent compared to the lowest value obtained after 45 days of transplanting. This might be due to the availability of host roots for colonization of VAM as symbiotic fungi. These results are in concurrence with the findings of Hayman (1980) who observed the increased root colonization throughout the growing season.

5.5.2.2 Isolation of DNA

The extraction of DNA from colonized roots reported by Henson *et al*. (1993) was tested for isolation of DNA. Bonito *et al*. (1995) reported that the addition of Chelex resin to the Tris-HCl buffer when the colonized roots were boiled resulted in increased amplification of the 550 bp AM rDNA product from

reaction mixture. He also reported that use of Chelex resin was critical for positive detection of *G. intraradices* in root samples of pepper and leek plants with low colonization (28 and 32% respectively).

5.5.2.3 Detection of AMF in cashew roots with PCR

PCR was performed for the amplification of 18S rDNA gene of *Glomus fasciculatum*. DNA amplification by PCR has been proposed as a technique for AM fungus identification (Simon *et al.* (1993); Wyss and Bonfante (1993). Simon *et al.* (1992a) reported that a portion of the small subunit rRNA gene (rDNA) specific for AM fungi can be amplified when a taxon-specific primer (VANSI) is paired with a universal primer (NS21).

In the present study, the 550-bp product was never detected in uninoculated, noncolonized control plants, even after two amplification rounds of 40 cycles each. Amplification of 550 bp was detected in colonized roots of tissue cultured plantlets.

5.6 Future prospects

Future line should be oriented towards the evaluation of more antibiotic for control of the endophytic and covert bacteria. Different ranges of fungicides should be evaluated for control of endophytic fungi identified. Enhancement of multiplication rate will supplement the vegetative propagation. More auxin treatments should be evaluated for induction of rooting of shoots derived from field plants.

Summary

SUMMARY

The investigations on "Management of recalcitrancy in *in vitro* cultures of cashew (*Anacardium occidentale* L.)" was conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Thrissur from 2006-2008. The main objectives of the study were to assess and manage the recalcitrance of *in vitro* cultures in cashew with respect to phenolic exudation, endophytic microbial contamination, induction of rooting with *Agrobacterium rhizogenes* and improvement of transplantation success by treatment with AMF-PGPR consortium. The salient findings of the study are as follows.

1. The protocol suggested by Dinakaran *et al*. 2003 was used for the isolation of endophytic bacteria from *in vitro* cultures of cashew. Serial dilution of ground sample in phosphate buffer revealed detection of one bacterium, named as KAU-EC1.
2. Among the 20 *in vitro* cultures of cashew, four of them turned index positive for medium during the first screening (after growing the cultures in antibiotic medium for one month). Dilution plating revealed two bacteria in each culture. These bacteria purified by subsequent streaking, were named as KAU-CC1 and KAU-CC2.
3. Cultural and morphological characteristics of the KAU-EC1, KAU-CC1 and KAU-CC2 were studied on nutrient agar media. KAU-EC1 produced large mucoid, opaque, circular convex colonies on nutrient agar medium. KAU-CC1 gave rise to circular, opaque yellow to creamy colonies. KAU-CC2 gave rise to circular, smooth, undulate, convex and yellow coloured colonies.
4. KAU-EC1, KAU-CC1 and KAU-CC2 were Gram negative and formed viscous threads after the KOH treatment. The cells were rod shaped, purple in colour.

5. The intrinsic antibiotic resistance pattern showed variable response to antibiotics. KAU-EC1 and KAU-CC2 were resistant to streptomycin, cefotaxime, gentamycin, ampicillin, nalidixic acid and tetracyclin at the highest concentration tested. KAU-CC1 was sensitive to streptomycin, cefotaxime and gentamycin and resistant to ampicillin, nalidixic acid and tetracycline.
6. Single colonies of pure cultures were stab inoculated in NA media in cryostorage vials and maintained under refrigerated conditions.
7. Total DNA was isolated from KAU-EC1, KAU-CC1 and KAU-CC2. The quantity of DNA in the three isolates varied from 241.3, 69.7 and 54.1 ng/µl respectively. The OD_{260}/OD_{280} ranged between 1.77-1.85 indicating good quality of DNA.
8. The amplified gene fragment obtained with the isolate KAU-EC1, KAU-CC1 and KAU-CC2 were eluted, cloned into pGEMT vector and competent *E. coli* cells were transformed with the ligated product. A combination of blue and white colonies was obtained after overnight incubation confirming successful transformation. Recombination efficiency range between 42-40% was observed for the three amplicons cloned.
9. Presence of insert was checked by PCR amplification of the cloned insert. Single amplified bands similar to the genomic DNA amplification were obtained in plasmids of white colonies. Plasmids isolated from blue colony could not produce any amplification.
10. The cloned insert of 16S rDNA genes of KAU-EC1, KAU-CC1 and KAU-CC2 were sequenced using T7 universal primer.
11. After vector screening of the sequences, theoretical analysis of the three sequences using Blastn was carried out.
12. KAU-EC1 showed 99 per cent homology with *Klebsiella pneumoniae* strain SA-D6-7 16S ribosomal RNA gene.

13. KAU-CC1 showed 98 per cent homology with *Pantoea agglomerans* strain XW123 16S ribosomal RNA gene.
14. KAU-CC2 showed 95 per cent homology with *Stenotrophomonas maltophila* strain H2S8 16S ribosomal RNA gene.
15. Three fungal species were detected 2, 4 and 6 weeks after culturing of nodal segments derived from field plants. The fungal species were identified as *Fusarium oxysporum*, *Fusarium moniliformae* and *Botryodiplodia theobromae*.
16. Phenol leached out into sterile water and liquid media (1/2 MS + 0.4 g glutamic acid) were estimated at different time periods.
17. Friedman two way analysis of variance of ranks was carried out to detect the differential leaching of phenol into liquid media and sterile water. The Friedman test statistic of phenol leached into liquid media was 5.16 at 15.1 per cent level of significance. The Friedman test statistic of phenol leached into sterile water was 14.04 at 0.3 per cent level of significance.
18. The highest mean rank score of phenol leached into liquid media was obtained for 0.2 per cent PVP followed by 0.5 per cent activated charcoal, followed by 100 mg ascorbic acid and control. Thus 0.2 per cent PVP is very effective to adsorb the phenols.
19. Survival and bud break per cent of nodal segments derived from field plants after 5-6 weeks culture was maximum during summer months especially April and lesser during June-July.
20. Surface sterilization of nodal segments derived from field plants with 0.1% $HgCl_2$ for 2 minutes was found to be optimum for culture establishment.
21. Among different basal media tested, maximum bud sprouting of forty five per cent was observed in ½ MS media whereas twenty per cent bud sprouting was observed in full MS media.

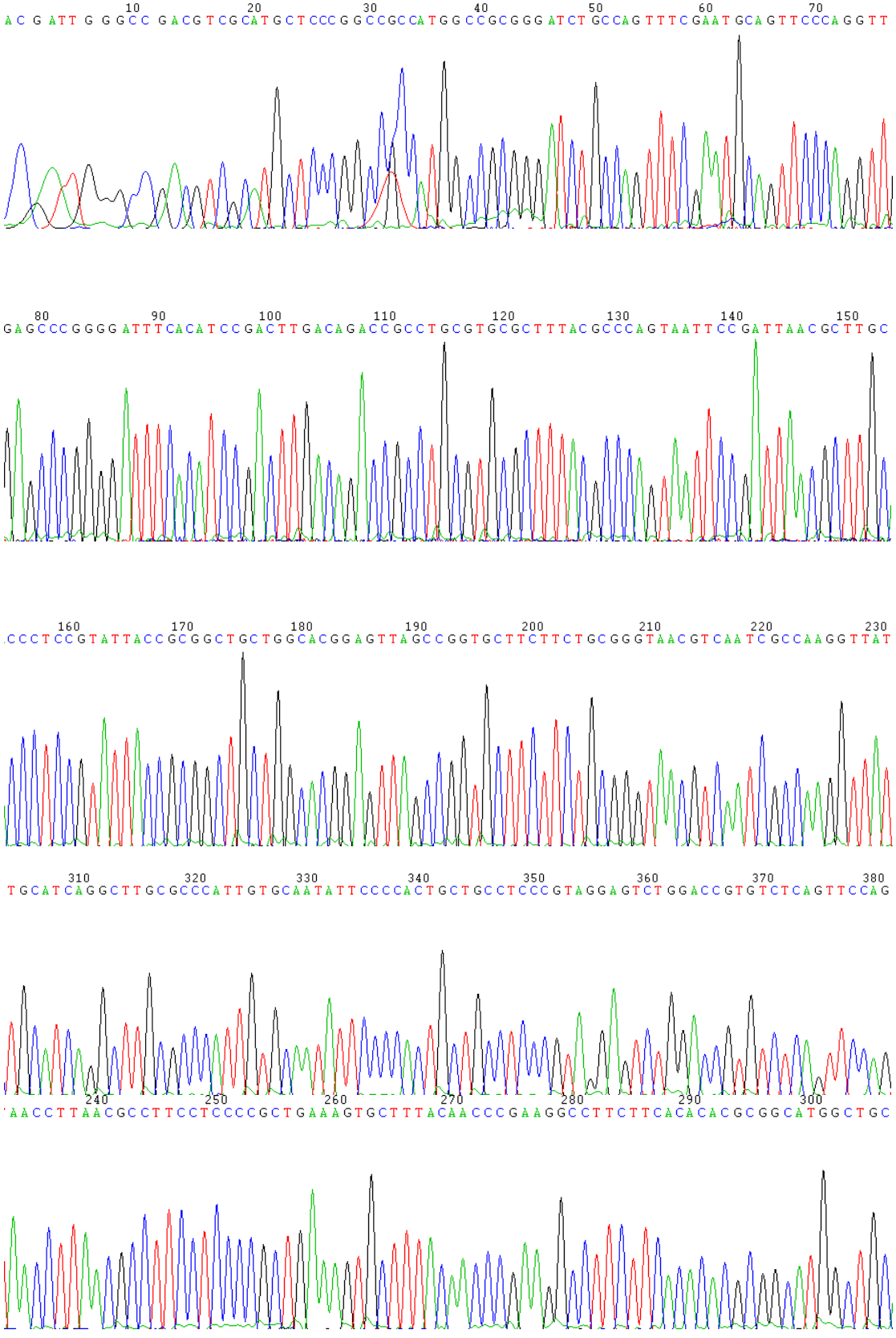
22. Among the different combination tested, maximum establishment of nodal segments were on MS medium supplemented with 400 mg l^{-1} glutamine and 0.2% PVP.
23. Maximum multiplication was obtained from nodal segments derived from field grown plants on MS with TDZ 0.45 µM, 400 mg l^{-1}glutamine and 0.2% PVP.
24. Elongated shoots obtained from nodal segments derived from field grown plants failed to produce roots in different media combinations tested.
25. Maximum seed germination (80%) was obtained on MS media supplemented with 3.5 mgl^{-1} kinetin and 1 mgl^{-1} NAA. Seeds were germinated within 15 days after inoculation.
26. Among the different combinations tested, the multiplication of shoot buds derived from nodal segments and cotyledonary nodes were more on MS medium supplemented with 1.5 mg l^{-1} BA, 1 mg l^{-1} IAA and 0.05% activated charcoal.
27. Considering the formation and elongation of shoot buds together maximum multiplication was obtained from MS medium supplemented with 2.5 mg l^{-1} BA, 1 mg l^{-1} IAA and 0.05% activated charcoal.
28. Maximum elongation of nodal and cotyledonary node derived shoots occurred on MS medium supplemented with 2 mg l^{-1} BA, 400 mg l^{-1} glutamic acid and 0.05% activated charcoal. Plantlets showed maximum length and vigor with dark green leaves in this medium.
29. Eighty per cent of rooting occured on half MS with 1 mg l^{-1} IBA and 1 mg l^{-1} IAA after pulse treatment with 24 mg l^{-1} IBA for 24 hrs.
30. Among the two *Agrobacterium rhizogenes* strains tested, the strain MTCC 2364 was able to induce normal roots in *Anacardium*

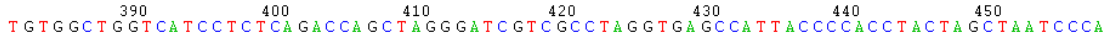
occidentale whereas, the strain MTCC 532 failed to induce normal roots by any of the transformation methods.

31. Rooting with *Agrobacterium rhizogenes* was influenced by the co-culture period. Shoot tips showed maximum response to the three-day co-culture. In general 25-30 days was taken for root induction. Thirty per cent rooting was observed.
32. The presence of 10 mM and 20 mM acetosyringone was not found to positively influence root induction with *Agrobacterium rhizogenes*.
33. Surface sterilized spores of *Glomus fasciculatum* were inoculated into rooting media before the initiation of rooting and after the initiation of rooting. There was no colonization after 30 days of inoculation of *Glomus* spores.
34. Twenty four hrs old cultures of *Bacillus subtilis* (10^8 cfu) was inoculated after the initiation of rooting and three days before transplanting. Basal portion of explants decayed after one week, due to higher multiplication rate of bacteria. There was no positive influence on the rooting of the inoculation of *Bacillus subtilis*.
35. The tissue cultured plantlets were inoculated with *Glomus fasciculatum* and *Bacillus subtilis* and their consortium before planting out. Maximum survival per cent (70.72%) was observed in treatment involving *Glomus fasciculatum* and *Bacillus subtilis* followed by treatment with *Bacillus subtilis* with 68.35% and then by *Glomus fasciculatum* with 66.68% survival against 50.22 per cent recorded for control.
36. Combined inoculation of *Glomus fasciculatum* and *Bacillus subtilis* induced maximum height and root growth of the tissue cultured plantlets.
37. Seventy per cent colonization was observed after 90 days of transplanting in the present study.

38. The addition of Chelex resin to the Tris-HCl buffer when the colonized roots were boiled resulted in increased amplification of the 550 bp AM rDNA product from the reaction mixture.
39. The 550 bp rDNA amplification product characteristic of AM fungi was consistently amplified from roots of tissue cultured plantlets colonized by *Glomus fasciculatum*. The 550 bp product was not detected from uninoculated, non-colonized control plants, even after two amplification rounds of 40 cycles each.

Electrogram of 16S rRNA sequences of KAU-EC1

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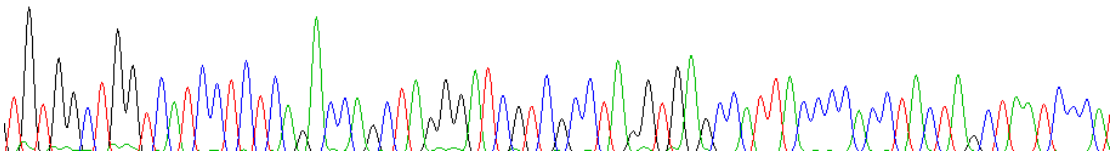


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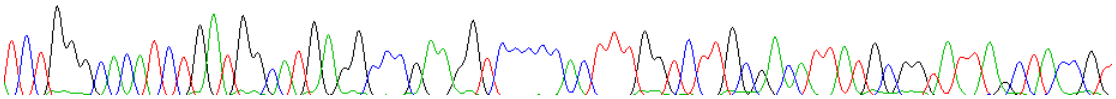

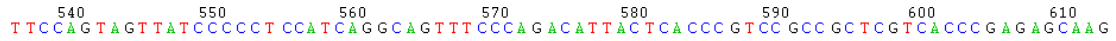
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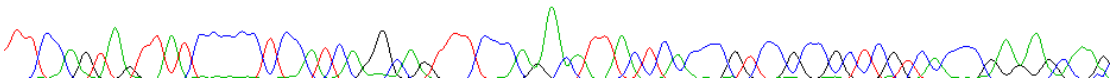


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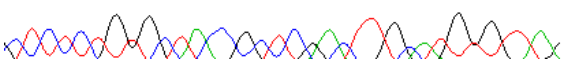
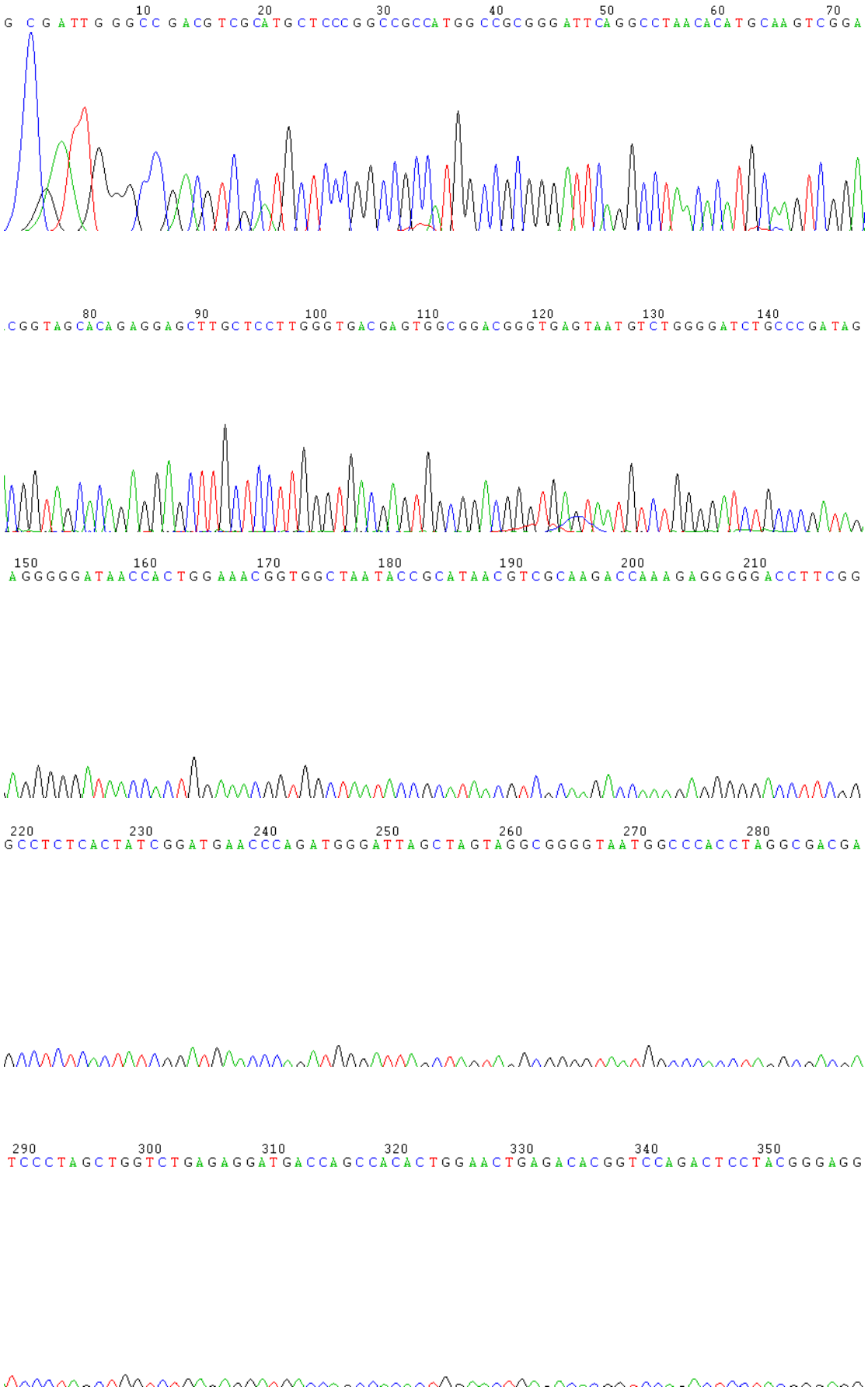
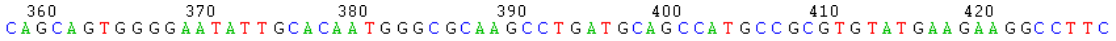


Electrogram of 16S rRNA sequences of KAU-CC1

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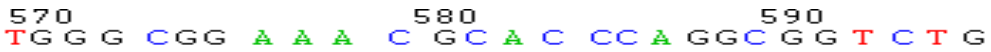
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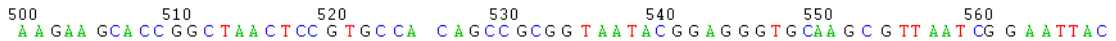
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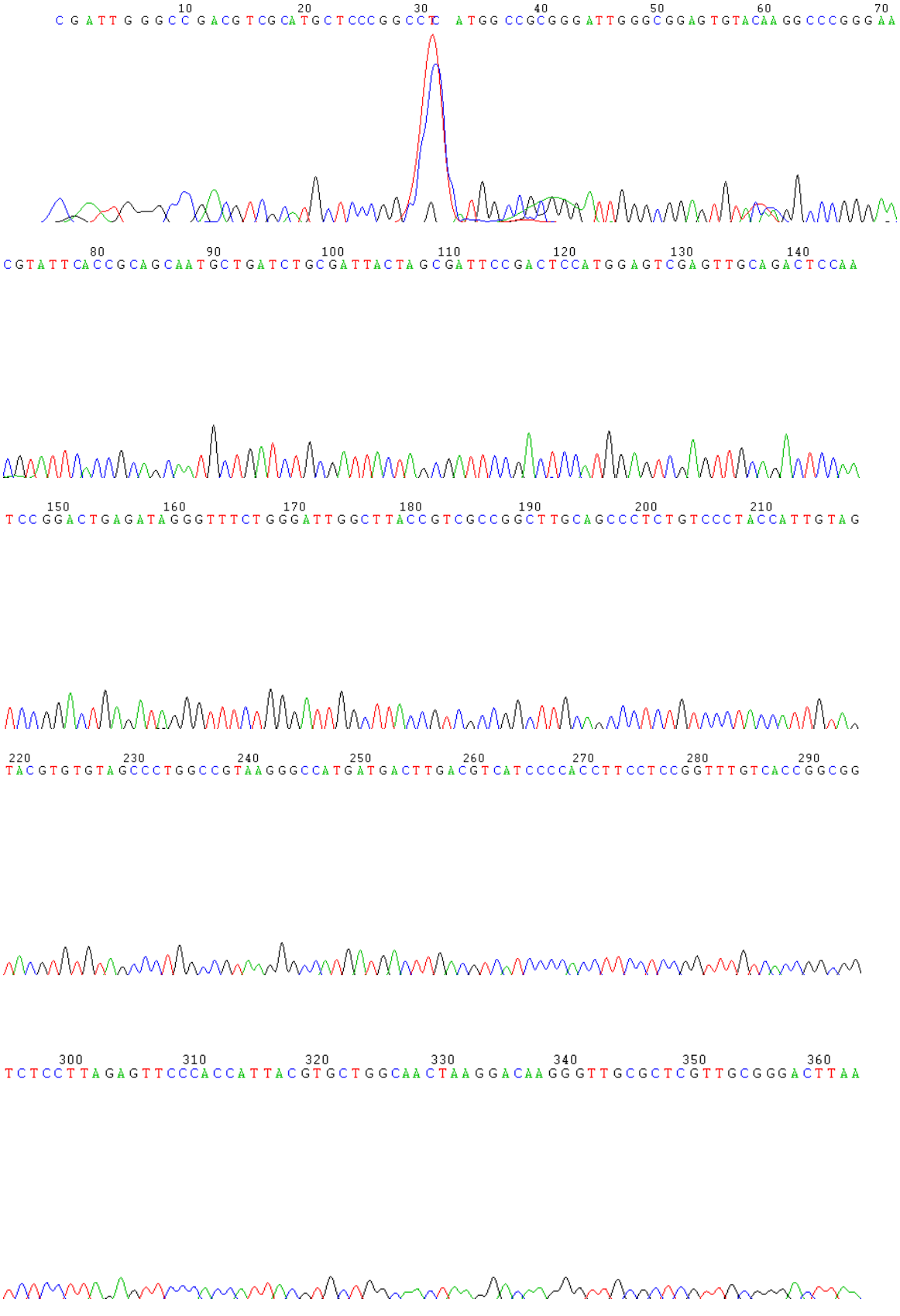
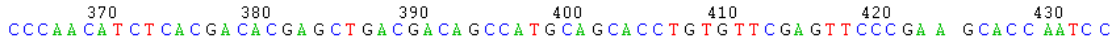
Electrogram of 16S rRNA sequences of KAU-CC2

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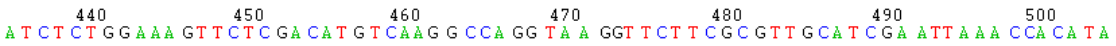
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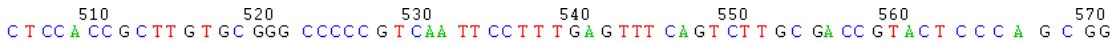
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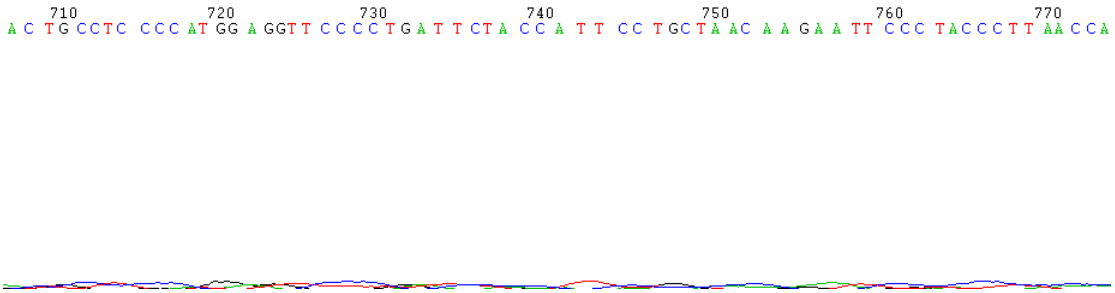
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890
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References

REFERENCES

Adholeya, A. and Cheema, G.S. 1990. Evaluation of VA mycorrhizal inoculation in micropropagated *Populus deltoids* Marsh clones. *Curr. Sci.* 59:1244-1247

*Agrolioni, M. and Gailians, F. 1977. Cashew Cultivation. Instituto Agonomico Per L' Othermare, p.168.

Aguilera-Gomez, L., Davies, F.T.Jr., Olalde-Portugal, V., Duray, S.A. and Phavaphutanon, L. 1999. Influence of phosphorus and endomycorrhiza (*Glomus intraradices*) on gas exchange and plant growth of while ancho pepper (*Capsicum annuum* L. cv. San Luis). *Photosynthetica* 36:441-449

Aguilero-Gomez, L.I., Ramirez–Moreles, P. and Frias–Hernandez, J.T. 1998. Influence of *Glomus fasciculatum* on physiology and growth of three kinds of maize. *Phyton-Int. J. Exp. Bot*. 62:101-102

Alarcon, A., Ferrera-Cerrato, R., Gonzalez-Chavez, M.C. and Villegas-Mantes, A. 2000. Arbuscular mycorrhizal fungi in runner dynamics and nutrition of strawberry plants cv. Fern obtained from *in vitro* culture. *Terra* 18(3):211-218

Al-Karaki, G.N. 1998. Benefit, cost and water-use efficiency of arbuscular mycorrhizal durum wheat grown under drought stress. *Mycorrhiza* 8:41-45

Allen, M.F. 2007. Mycorrhizal fungi: Highways for water and nutrients in arid soils. *Vadose zone J.* 6:291-297

Allen, M.F., Moore, T.S. and Christensen, M. 1980. Phytophormone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae I. cytokinin increases in the host plant. *Can. J. Bot*. 58:371-374

Allen, M.F., Moore, T.S., Christinsen, M. and Stanton, N. 1979. Growth of vesicular-arbuscular mycorrhizal and non-mycorrhizal *Boviteloua gracilis* in a defined medium. *Mycologia* 71:666-669

Aliyu, O.M. and Awopetu, J.A. 2005. *In vitro* regeneration of hybrid plantlets of cashew (*Anacardium occidentale* L.) through embryo culture. *Afr. J. Biotechnol*. 4(6):548-553

Altschul, S.F., Thomas, L.M., Schaffer, A.A., Zhang, J., Zhang, S., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25:3389-3402

Ananthakrishnan, G., Ravikumar, R., Girija, S. and Ganapathi, A. 2002. *In vitro* adventitious shoot formation from cotyledon explants of cashew (*Anacardium occidentale* L.). *Scientia Horti*. 93(3-4):343-355

Ananthakrishnan, G., Ravikumar, R., Anand, P.R., Vengadesan, G. and Ganapathi, A. 1999. Induction of somatic embryogenesis from nucellus-derived callus of *Anacardium occcidentale* L. *Sci. Horti*. 79:91-99

Anandaraj, M. and Sarma, V.R. 1994 a. Effect of vesicular arbuscular mycorrhiza on rooting of black pepper (*Piper nigrum* L.). *J. Spices Aromatic Crops* 3:39-42

Anandaraj, M., Venugopal, M.N. and Sarma, Y.R. 1996. *Biological Control of Diseases of Spice Crop*. Annual Report 1995-96, Indian Institute of Spice Research, Calicut, Kerala, 71p.

Armstrong, J.L., Kanudesem, G.R. and Seidler, R.J. 1987. Microsm method to assess survival of recombinant bacteria associated with plants and herbivorous insects. *Curr. microbiol.* 15:229-232

Ascenso, J.C. 1986. Potential of the cashew crop. *Agric. Intl.* 38:368-371

Atkinson, D. and Davidson, A.W. 1972. The effects of phosphorus deficiency on water content and response to drought. *New Phytol*. 72:307-313

Azcon-Aguilar, C., Barcelo, A., Vidal, M.T. and Vina, G.D. 1992. Further studies on the influence of mycorrhizae on growth and development of micropropagated avocado plants. *Agronomie* 12:837-840

Ball, E.A. 1950. Differentiation in a callus cultures of *Seqoia sempervirence*. *Growth* 14:295-325

Barea, J.M. and Azcon-Aguilar, C. 1982. Production of plant growth regulating substances by vesicular-arbuscular mycorrhiza *Glomus mosseae*. *Appl. Environ. Microbiol.* 43:810-813

Bashan, V. 1986. Field dispersal of *Pseudomonas syringae*. pv. tomato *Xanthomonas campestris* pv. *vesicatoria* and *Alternaria macrospora* by

animals, peoples, birds, insects, mites, agrl. Tools, aircraft, soil particles and water sources. *Can. J. Bot*. 64:276-281

Bassil, N.V., Proebsting, W.M., Moore, L.W. and Lightfoot, D.A. 1991. Propagation of hazelnut stem cuttings using *Agrobacterium rhizogenes. Hort. Sci.* 26:1058-1060

Behrens, R. 1998. About the spacing of cashew nut trees. In: Proceedings of International Cashew and Coconut Conference -1997. Dar es Salaam, pp. 48-52

Bell, C.R., Dickie, G.A., Harvey, W.L.G. and Chan, J.W.Y.F. 1995. Endophytic bacteria in grape vine. *Can. J. Microbiol.* 41:46-53

Berg, G. and Hallmann, J. 2006. Control of plant pathogenic fungi with bacterial endophytes. In: Schulz, B.J.E., Boyle, C.J.C. and Sieber, T.N. (eds.), *Microbial Root Endophytes*. Springer-Verlag, Berlin, pp. 53-69.

Berta, G., Fusconi, A., Trotta, A. and Scannerini, S. 1990. Morphogenetic modifications induced by the mycorrhizal fungus *Glomus* strain E3 in the coat system or *Allium porum*. *New Phytol*. 114:207-215

Berta, G., Trotta, A., Fusconi, A., Hooker, J., Munro, M., Atkinson, D., Givanneth, M., Morini, S., Fortuna, P., Tisserant, B., Gianinazz-Pearson, V. and Gianinazzi, S. 1995. Arbuscular mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera* L. *Tree Physiol.* 15:281-293

Bessa, A.M.S. 1990. *In vitro* propagation of cashew (*Anacardium occidentale* L.) by nodal culture. In: *Abstracts of the VIIth International Congress Plant Tissue Cell Culture*, Amsterdam, June 24-29, Abstract D 1-8.

Bessa, A.M.S. and Sardinha, R.M.A. 1994. *In vitro* cashew (*Anacardium occidentale* L.) propagation from callus culture. In: *Abstracts of the VIIIth international congress on plant tissue and cell culture*. IAPTC Firenze Italy, June 12-17, p. 23.

Bethlenfalvay, G.J. and Newton, W.E. 1991. Agro-ecological aspects of the mycorrhizal, nitrogen fixing legume symbiosis. In: Keister, D.L. and

Cregan, P.B. (eds.), *The Rhizosphere and plant growth*. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 349-354.

Bhatia, I.S., Uppal, S. and Bajaj, K.L. 1972. Histochemical study suggested that the higher amounts of phenols and lipids observed in mycorrhizal plants might be the contribution of the fungal structures. *Indian Phytopath* 25: 231

Binns, A.N. and Thomashow, M.F. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. *A. Rev. Microbiol.* 42:575-606

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523

Blal, B. and Gianinazzi-Pearson, V. 1988. Interest of endomycorrhizae for the production of micropropagated oil palm clones. In: Robson, A.D. and Barrow, N.J. (eds.), *Proceedings of the Second European Symposium on Mycorrhizae*; 14-20 August, 1988; Prague, Czechoslovakia, pp. 115-127.

Blal, B., Morel, C., Gianinazzi-Pearson, V., Fardeau, J.C. and Gianinazzi, S. 1990. Influence of vesicular-arbuscular mycorrhizae on phosphate fertilizer efficiency in two tropic acid soils planted with micropropagated oil palm (*Elaeis guineensis* Jacq.). *Biol. Fert. Soils* 9:43-48

Boggetti, B., Jasik, J. and Mantel, S. 1999. *In vitro* multiplication of cashew (*Anacardium occidentale* L.) using shoot node explants of glasshouse raised plants. *Pl. Cell Rep.* 18:456-461

Bohlool, B.B., Ladha, J.K., Garrity, D.P. and George, T. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. *Pl. Soil* 141:1-11

Bolam, N.S., 1987. Effects of vesicular arbuscular mycorrhiza on the availability of iron phosphates to plants. *Pl. and Soil* 99: 401-410

Bolton, G.W., Nester, E.W. and Gordon, M.P. 1986. Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. *Science* 232:983-985

Bonito, R.D., Elliott, M.L. and Jardin, D.E.A. 1995. Detection of an arbuscular mycorrhizal fungus in roots of different plant species with the PCR. *Appl. Environ. Microbiol.* 61(7):2809-2810

Bopaiah, B.M. and Khader, A.K.B. 1989. Effect of biofertilizers on growth of black pepper (*Piper nigrum* L.). *Indian J. agric. Sci.* 59:682-683

Borrowska, B. 2002. Growth and photosynthetic activity of micropropagated strawberry plants inoculated with AMF and growing under drought strees. *Acta Physiologiae Pl.* 24:365-370

Bose, T.K., Das, P. and Maiti, G.G. 1998. *Trees of the world, Volume I.* Regional Plant Resource Centre, Bhuvaneswar, Orissa, p-53.

Bose, T.K., Maiti, G.G., Sangal, D. and Sadhu, M.K. 2006. *Dictionary of Horticu;lture* Volume 1. Naya Udyou, Kolkata, pp. 320-321.

Boucher, A., Dalpo, Y. and Charest, C. 1999. Effect of arbuscular mycorrhizal colonization of four species of *Glomus* on physiological responses of maize. *J. Pl. Nutr.* 22:783-797

Boxus, P.H. and Terzi, J.M. 1987. Big losses due to bacterial contaminations can be avoided in mass propagation scheme. *Acta Hortic.* 212:91-93

Bucher, M. 2006. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. *New Phytologist* 173:11-26

Budi, S.W., Gianinazzi, S. and Blal, B. 1999. Surface sterilization of *Glomus mosseae* sporocarps for studying endomycorrhization *in vitro*. *Mycorrhiza* 9(2):65-68

Calderan, L.D., Gomez, L., Blanco, F. and Uribe, L. 2001. Inoculation with *Glomus manihotis* on growth and development of *in vitro* propagated cassava plants during acclimatization. *Agronomia Costarricense* 24(2):25-29

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clement, C. and Ait Barka, E. 2005a. Endophytic colonization of *Vitis vinifera* L. by a plant growth promoting bacterium, *Burkholdiria* sp. strain PsJN. *Appl. Environ. Microbiol.* 71:1685-1693

Capellades, M., Fantarnau, R., Carulla, C. and Debery, P. 1990. Environment influences anatomy of stomata and epidermal cells in tissue cultured *Rosa multiflora*. *J. Am. Soc. Hort. Sci* 115:141-145

Caravaca, F., Diaz, E., Barea, J.M., Azcon–Aguilar, C. and Roldan, A. 2003. Photosynthetic and transpiration rates of *Olea europaea* Sub. Sylvestris and *Rhamnus lycioides* as affected by water deficit and mycorrhiza. *Biologia P1*. 46:637-639

Cardarelli, M., Mariotti, D., Pomponi, M., Spano, L., Capone, I. and Costantino, P. 1987. *Agrobacterium rhizogenes* t-DNA genes capable of inducing hairy root phenotype. *Mol. Gen. Genet*. 209:475-480

Cardarelli, M., Spano, L., Paolis, D.A., Mauro, M.L., Vitali, G. and Constantino, P. 1985. Identification of the genetic locus responsible for non polar root induction by *Agrobacterium rhizogenes* 1855. *Pl. Mol. Biol*. 5:385-391

Caris, C., Hoerdt, W., Hwkins, H.J., Roenheld, V. and George, E. 1998. Studies on the iron transport by arbuscular mycorrhizal hyphae from soil to peanut and sorghum plants. *Mycorrhiza* 8:35-39

Cassells, A.C. 1991. Problems in tissue culture: culture contamination. In: Debergh, P.C. and Zimmerman, R.H. (eds.), *Micropropagation*. Kluwer Academic Publishers, Dordrecht, pp. 31-44.

Cassells, A.C. 2000. Contamination detection and elimination in plant cell culture. In: Spier, R. E. (ed.), *Encyclopedia of Cell Technology*, John Wiley, New York, vol.2 pp. 577-586.

Chabra, M.L. and Jalali, B.L. 1995. Effect of vesicular arbuscular mycorrhiza on utilization of rock phosphate in pigeon pea. In: Adholeya, A. and Singh, S. (eds.), *Mycorrhiza; biofertilizer for the future* Proceedings of the third national conference on mycorrhiza, 12-17 September, 1994; Tata Energy Research Institute, New Delhi, pp. 413-415.

Chandha, K.L. 1991. Presidential Address. Natl. Group discussion of cashew Res. workers held at CPCRI, Kasargod, Kerala, 30-31 August and 1 September 1991.

Chen B.D., Li. X.L., Tao H.Q. Christie P. and Wong M.H. 2003. The role of arbuscular mycorrhiza in zinc uptake by red cloves growing in calcarous soil spiked with various quantities of zinc. *Chemosphere* 50:839-846

Chilton, M.J., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F. and Tempe, J. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. *Nature* 295:432-434

Chopade, B.A., Sachdev, D.P., Agarwal, V.S., Verma, P., Shouche, Y.S. and Dhakephalkar, P.K. 2007. Diversity of Acinetobacter, *Pseudomonas* species and diazotrophs population in the rhizosphere of wheat and their plant growth promoting properties. Submitted to Molecular Biology Unit, National Centre for Cell Sciences, Ganeshkhind, Pune University Campus, Pune, Maharashtra.

Christopher R.B. and Tony J.V. 2008. Maize drought tolerance: Potential improvements through arbuscularmycorrhizal symbiosis. *Field Crops Res.* 108:14-31

Citovsky, V., Mc leam, B.G., Greene, E., Howard, E., Kuldau, G., Yuonne, T., Zupan, J. and Zambryski, P. 1991. *Agrobacterium*-plant cell interaction of vir genes and T-DNA transfer. In: Verma, D.P.S. (ed.), *Molecular Signals in Plant Microbe communications*. CRC press, London, pp. 169-199.

Clark, R.B. and Zeto, S.K. 1996. Growth and root colonization of mycorrhizal maize grown on acid and alkaline soil. *Soil Biol. Biochem* 28:1505-1511

Compant, S., Duffy, B., Nowak, J., C.l.C. and Barka, E.A. 2005a. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71:4951-4959

Comprubi, A, Estuan, V., Valvet, C. and Pera, J, 1990. Infectivity and effectivity of *Glomus mosseae* in four different species of medicinal plants. *Symbiosis* 9:305-301

Cook, A.A., Larson, R.H. and Walker, J.C. 1952. Relation of black rot pathogen to cabbage seed. *Phytopathology* 42:316-320

Cooke, D.L., Waites, W.M. and Leifert, C. 1992. Effect of *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* on plant tissue cultures of *Aster*, *Cheiranthus*,

Delphinium, *Iris* and *Rosa*, disease development as a result of latent infection *in vitro*. *J. Pl. Dis. Protect*. 99:469-481

Daft, G.C. and Leben, C. 1972. Bacterial blight of soybeans: seedling infection during and after emergence. *Phytopathology* 62:1167-1170

Damiano, C., Archilletti, T., Caboni, E., Lauri, P., Falasca, G., Mariotti, D. and Ferraiolo, G. 1995. *Agrobacterium* mediated transformation of Almond: *In vitro* rooting through localized infection of *A. rhizogenes* W.T. *Acta Hort*. 392:161-169

Danneberg, G., Latus, C., Zimmer, W., Hundeshagen, B., Schneider-Poetsch, H.J. and Bothe, H. 1992. Influence of vesicular-arbuscular mycorrrhiza on phytohormone balances in maize (*Zea mays* L.) *J. Pl. Physiol*. 141:33-39

Das, S., Jha, T.B. and Jha, S. 1996. *In vitro* propagation of cashew nut. *Pl. Cell Rep*. 15:615-619

Davies, F.T.Jr., Potter, J.R. and Linderman, R.G. 1993. Drought resistance of mycorrhizal pepper plant independent of leaf P concentration response in gas exchange and water relations. *Physiol. Pl*. 87:45-53

Debergh, P., Harbaoui, V. and Lemeur, R. 1981. Mass propagation of globe artichoke (*Cynara scolymus*): evaluation of different hypotheses to overcome vitrification with special reference to water potential. *Physical P1.Path* 53:181-187

Declerck, S., Devos, B., Delvaux, B. and Plenchette, C. 1994. Growth response of micropropagated banana plants on VAM inoculation. *Fruits* 49:103-109

Dedrichs, C. and Moawad, A.M. 1993. The potential of VA mgcorrhizae for plant nutrition in the tropics. *Angew Bot*. 67:91-96

De Fossard, R.A. 1976. *Tissue culture for plant propagators*. Department of Continuing Education, University of New England Printery, Armeidale, Australia.

Dehne, H.W. and Schonbeck, F. 1979. The influence of endotropic mycorrhiza on plant diseases I. colomization of tomato plant by *Fusarium oxysporum* fsp. lycopersici. *Phytopathology* 2(95):105-11

Dell'Amico, J., Torrecillas, A., Rodriguez, P., Morte, A. and Sanchez-Blanco M.J. 2002. Responses of tomato plants associated with the arbuscular mycorrhizal fungus *Glomus clarum* during drought and recovery. *J. agric. Sci.* 138:387-393

Derakshani, M., Lukow, T. and Liesack, W. 2001. Novel bacterial lineages at the (sub) division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Appl. Environ. Microbiol*. 67:623-631

Diedrichs, C. and Moawad, A.M. 1993. The potential of VA mycorrhizae for plant nutrition in the tropics. *Angew Bot*. 67:91-96

Dinakaran, D., Arjunan, C. and Karthikeyan, G. 2003. Biological control of diseases. Tamil Nadu Agricultural University, Coimbatore, 28p.

Directorate of Cashew and Cocoa Development. 2008. Statistics.

Dojka, M.A., Harris, J.K. and Pace, N.R. 2000. Expanding the known diversity and environmental distribution of a cultured phytogenetic division of bacteria. *Appl. Environ. Microbiol*. 66:1617-1621

Dommisse, E.M., Leung, D.W.M., Shaw, L.M. and Conner, A.J. 1990. Onion is a monocotyledonous host for *Agrobacterium*. *Pl. Sci.* 69:249-257

Dong, Z., Canny, M.J., McCully, M.E., Roboredo, M.R., Cabadilla, C.F., Ortega, E. and Rodes, R. 1994. A nitrogen fixing endophyte of sugarcane stems. *Pl. Physiol.* 105:1139-1147

Donnelly, D.J. and Vidaver, W.E. 1984. Leaf anatomy of red raspberry transferred from culture to soil *J. Am. Soc. Hort. Sci*. 109:172-176

D'Silva, I. 1991. Micropropagation of *Ailanthus* and *Anacardium occidentale* through tissue culture. Ph.D. thesis, Mangalore University, India.

D'Silva, I and D'Souza, L. 1992a. In vitro propagation of Anacardium occidentale L. *Pl. Cell Tiss. Organ Cult*. 29:1-6

D'Silva, I. and D'Souza, L. 1992b. *In vitro* bud proliferation of *Anacardium occidentale* L. *Beitr Biol Pflanzen*, 67:273-279.

D'Silva, I. and D'Souza, L. 1993. Controlling contamination and browning of *in vitro* cultures in cashew. *J. Plantn. Crops* 21(1):22-29

D'Souza, L., D'Silva, I., Mallaya, S., Augustine, A.C., Rajendra, K., Kulkarni, K.R., Cardoza, V. 1996. *Anacardium occidentale* L. (Cashewnut). In: Bajaj, Y.P.S. (ed.), *Biotechnology in agriculture and forestry*. Springer and Verlag, New York, pp. 31-49.

D'Souza, L., Hedge, S., Augustine, A.C., Rajendra, K., Cardoza, V., Kulkarni, K. and D' Silva, I. 1999. Cashew (*Anacardium occidentale* L.) is a useful but hard nut to crack. In: Ravishankar, G.A. and Venkataraman, L.V. (eds.), *Biotechnological Applications of Plant Tissue and Cell Culture*. Oxford and IBH Publishing Co. Ltd., New Delhi, Calcutta, pp. 174-179.

Elliot, C. 1951. *Manual of Bacterial Plant Pathogens*. 2nd rev. ed. Chronica Botanica, Waltham, Mass.

Elvira-Recuenco, M. and Vuurde, V.J.W.L. 2000. Natural incidence of endophytic bacteria in pea cultivars under field conditions. *Can. J. Microbiol.* 46:1036-1041

Estaun, V., Calvet, C. and Camprubi, A. 1994. Arbuscular mycorrhizae and growth enhancement of micropropagated *Prunus* rootctock in different soilless potting mixes. *Agricultural and Food Sci. in Finland* 3:263-267

Estaun, V., Calvet, C., Camprubi, A. and Pinochet, J. 1999. Long-term effects of nursery starter substate and AM inoculation of micropropagated peach X almond hybrid root stock GF677. *Agronomie* 19:483-489

Esther, O.U. and Mark, D.L. 2007. Rhizotron studies on *Zea mays* L. to evaluate biocontrol activity of *Bacillus subtilis*. *World J. Microbiol. Biotechnol*. 24(2):269-274

Estrada–Luna, A.A. and Davies, F.T. 2003. Arbuscular mycorrhizal fungi influence water relations, gas exchange, abscisic acid and growth of microporpagated chile ancho pepper (*Capsicum annuum*) plantlets during acclimatization and post acclimatization. *J. Pl. Physiol* 160:1073-1003

Estruch, J.J., Scheli, J. and Spana, A. 1991. The protein encoded by the *rol* B, plant oncogenic hydrolyses indole-glucosides. *EMBO J.* 10:3125-3128

Faber, B.A., Zasoski, R.J., Munns, D.N. and Schakel, K. 1991. A method for measuring hyphal uptake in mycorrhizal plants. *Can J. Bot*. 69:87-94

Falasca, G., Reverberi, M., Lauri, P., Caboni, E., De Stradis, A. and Altamura, M.M. 2000. How *Agrobacterium rhizogenes* triggers de novo root formation in a recalcitrant woody plant: an integrated histological, ultrastructural and molecular analysis. *New Phytologist* 145(1):77-93

Farrant, J.M., Berjak, P. and Myoock, D.J. 1990. *In vitro* propagation of *Anacardium occidentale* L. (Cashew). In: *Abstracts of the VII International Congress on Plant Tissue Cell Culture*, Amsterdam, June 24-29, Abstract A 3-65.

Fernandez, K., Fernandez, F., Gonzalez, M.E., Perez, E., Mirabal, L. And Pazos, M. 2002. *In vitro* mycorrhization of coffee plantlets: is it a fact? *Cultivar Tropicales* 23(3):47-52

Fogher, G., Maggioni, L. and Cort, P. 1986. Effect of inoculation with *Glomus mosseae* on plantlets of garlic (*Allium sativum*) from callus. *Annal della facolla de Agraria* 26:9-14

Fortuna, P., Citernesi, S., Morini, S., Vitagliano, C. and Giovanetti, M. 1996. Influence of arbuscular mycorrhizae and phosphate fertilization on shoot apical growth micropropagated and plum rootstocks. *Tree Physiol.* 16:757-763

Fortuna, P., Morini, S. and Giovanetti, M. 1998. Effects of arbuscular mycorrhizal fungi on *in vitro* root initiation and development of micropropagated plum shoots. *J. Hort. Sci. and Biotechnol.* 73:19-28

Fox, R.T., Manners, J.G. and Myers, A. 1971. Ultra structure of entry and spread of *Erwinia carotovora* var. *atroseptica* into potato tubers. *Potato Res.* 14:61-73

Franks, A., Ryan, P.R., Abbas, A., Mark, G.L. and O'Gara, F. 2006. *Molecular Tools for Studying Plant Growth-Promoting Rhizobacteria (PGPR): Molecular Techniques for Soil and Rhizosphere Microorganisms*. CABI Publishing, Wallingford, Oxfordshire, UK

Frommel, M.I., Nowak, J. and Lazarovits, G. 1991. Growth enhancement and developmental modification of *in vitro* grown potato (*Solanum tuberosum*

sp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. *Pl. Physiol.* 96:928-936

Gamborg, O.L., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell. Res.* 50:151-158

Ganz, T.R., Kailis, S.G. and Abbott, L.K. 2002. Mycorrhizal colonization and its effect on growth, phosphorus uptake and tissue phenolic content in the European Olive (*Olea europaea* L.) *Adv. Hort. Sci.* 16:109-116

Ganzalez-Chavez, C., D'Haen, J., Vangronsveld, J. and Docdd, J.C. 2002. Copper sorption and accumulation by the extra radical mycelium of different *Glomus* spp (arbuscular mycorrhizal fungus isolated from the same polluted soil. *Pl. Soil* 240:287 -297

Gardner, J.M., Feldman, A.W. and Zablotowicz, R.M. 1982. Identity and behaviour of xylem-residing bacteria in rough lemon roots of Florida citrus trees. *Appl. Environ. Microbiol.* 43:1335-1342

Gaur, A., and Adholeya, A. 2004. Prospects of arbuscular mycorrhizal fungus in phytoremediation of heavy metal contaminated soils. *Curr. Sci.* 86:528-533

Gelvin, S.B. 1990. Crown gall disease and hairy root disease a sledge hammer and a trackhammer. *Pl. Physiol.* 92:281-285

Gerdemann, J.W. and Nicolson, T.H. 1963. Spores of mycorrhizal *Endogone* species extracted from soil by wet-sieving and decanting. *Trans. Br. Mycol. Soc.* 46:234-244

Gianinazzi-Pearson, V., Dumas-Gaudat, E., Gollot, A., Tahiri-Alaoui, A. and Gianinazzi, S. 1996. Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. *New phytol.* 133:45-57

Gildon, A. and Tinker, P.B. 1983. Interactions of vesicular-arbuscular mycorrhizal infection and heavy metals in plants. I. The effects of heavy metals on the development of vesicular arbuscular mycorrhizas. *New Phytol.* 95:247-261

Godwin, I., Todd, G., Ford-Llyod, B. and New burg, H.J. 1991. The effects of acetosyringone and pH on *Agrobacterium* mediated transformation vary according to plant species. *Pl. Cell Rep.* 9:671-675

Gogate, S.S. and Nadgauda, R.S. 2003. Direct induction of somatic embryogenesis from immature zygotic embryo of cashew nut (*Anacardium occidentale* L.). *Scientia Horti*. 97 (1):75-82

Goicoechea, N., Doleza, K., Antolin, M.C., Strand, M. and Sanchez-Diaz, M. 1995. Influence of mycorrhizae and *Rhizobium* on cytokinin content in drought stressed alfalfa. *J. Exp. Bot*. 46:1543-1549

Gonzalez-Chavez, C., D'Haen, J., Vangronsveld, J. and Dodd, J.C. 2002. Copper sorption and accumulation by the extra radical mycelium of different *Glomus* spp (arbuscular mycorrhizal fungi) isolated from the same polluted soil. *Pl. Soil* 240:287-297

Gosal, S.K., Gupta, R.P. and Gosal, S.S. 2001. *In vitro* establishment of VAM in roots of mciropropagated sugarcane. *Ann. Agric. Bio. Res*. 6 (1):19-22

Grandmaizon, J., Olah, G.M., Calsteren, V.M.R. and Furlan, V. 1993. Characterization and localization of plant phenolics likely involved in the pathogen resistance expressed by endomycorrhizal roots. *Mycorrhiza* 3:155-164

Graham, J.H., Leonard, R.T. and Menge, J.A. 1981. Membrane-mediated decrease in root exudation responsible for phosphorus inhibition of vesicular arbuscular mycorrhizae formation. *Pl. Physiol*. 68:534-552

Granger, R.L., Planchette, C. and Fortin, J.A. 1983. Effect of vesicular arbuscular (VAM) endomycorrhizal fungus (*Glomus epigaeum*) on the growth and leaf mineral content of two apple clones propagated *in vitro*. *Can. J. Pl. Sci*. 63:551-555

Grotkass, C., Hutter, I. and Feldmann, F. 2000. Use of arbuscular mycorrhizal fungi to reduce weaning stress of micropropagated *Baptisia tinctoria* (L.). R. Br. *Acta Hort*. 530:305-311

Grout, H.W.W. and Aston, M.J. 1977. Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. *Ann. Bot*. 42:993-995

Guillemin, J.P., Gianinazzi, S., Gianinazzi-Pearson, V. and Marchal, J. 1994. Contribution of arbuscular mycorrhizas to biological protection of

micropropagated pineapple (*Ananas comosus* (L.) Merr.) against *Phytophthora cinnamomi* R and S. *Agric. Sci.* Finland 3:241-251

Guillemin, J.P., Gianinazzi, S. and Trouvelot, A. 1992. Screening of VA endomycorrhizal fungi for establishment of micropropagated pineapple plants. *Agronomie* 12:831-836

Guo, V., George, E. and Marshner, H. 1996. Contribution of an arbuscular mycorrhizal fungus to uptake of Cadmium and Nickel in bean by maize plants. *P1. Soil* 184:195-205

Guttag, A.J.P. 1976. Impact of de-icing salts upon endo mycorrhizae of roadside sugar maple. *Soil Sci. Soc. Am. J.* 40:952-954

Habte, M. 1990. Strategies for the production of infected root-based VA mycorrhizal inocula. *Mycorrhiza News* 2:1-2

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F. and Kloepper, J.W. 1997. Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* 43:895-914

Hardic, K. and Layton, L. 1981. The influence of VA mycorrhizae on growth and water relations of red clover in phosphate deficient soil. *New Phytol* 89:599-608

Harley, J.L. and Smith, S.E. 1983. *Mycorrhizal symbiosis*. Academic Press, London, 288 p.

Hashidoko, Y., Itoh, E., Yokota, K., Yoshida, T. and Tahara, S. 2002. Characterization of five phyllosphere bacteria isolated from *Rosa rugosa* leaves and their phenotypic and metabolic properties. *Bioscience, Biotechnol. and Biochem*. 66(11):2474-2478

Hatta, M., Beyl, C.A., Garton, S. and Diner, A.M. 1996. Induction of roots on jujube softwood cuttings using *Agrobacterium rhizogenes*. *The J. Hort. Sci.* 71(6):881-886

Hawes, M.C., Smith, L.Y. and Howarth, A.J. 1988. *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Mol. Plant-Microbe Interaction* 1:182-190

Hayman, D.S. 1980. Mycorrhizae and production of crops. *Nature* 287:487-488

*Hedge, M. 1988. Ph.D. thesis, Tamil Nadu Agricultural University, Coimbatore, India, p. 169.

Hedge, M. and Kulasekaran, M. 1994. Proceedings of the XI Symposium on Plantation Crops, Tamil Nadu Agricultural University, Coimbatore, India.

Hedge, M., Kulasekaran, M., Shanmugavelu, K.G. and Jayasankar, S. 1990. *In vitro* culture of cashew seedlings and multiple plantlets from mature cotyledons. *Indian Cashew J*. 20:19-24

Hedge, M., Kulasekaran, M., Jayasankar, S. and Shanmugavelu, K.G. 1991. *In vitro* embryogenesis in cashew (*Anacardium occidentale* L.). *Indian Cashew J*. 21:17-25

Henson, J.M., Goins, T., Grey, W., Mathre, D.E. and Elliot, M.L. 1993. Use of polymerase chain reaction to detect *Gaeumannomyces graminis* DNA in plants grown in artificially and naturally infested soil. *Phytopathology* 83:283-287

Hernalsteens, J.P., Thia-Toong, L., Schell, J. and Montagu, V.M. 1984. An *Agrobacterium* transformed cell culture from the monocot *Asparagus officinalis*. *EMBO J*. 3:3039-3041

Hernandez-Sebastia, C., Piche, Y. and Desjardins, Y. 1999. Water relations of whole strawberry plantlets *in vitro* inoculated with *Glomus intraradices* in a tripartite culture system. *Pl. Sci*. 143:81-91

Hobbs, S.L.A., Jackson, J.A. and Mahon, J.D. 1989. Specificity of strain and genotype in the susceptibility of pea to *Agrobacterium tumefaciens*. *Pl. Cell Rep*. 8:55-58

Holland, M.A. and Polacco, J.C. 1994. PPFMs and other covert contaminants: Is there more to plant physiology than just plant ? *Annu. Rev. Pl. Physiol. Pl. Mol. Biol.* 45:197-209

Holliday, P. 1989. A Dictionary of Plant Pathology. Cambridge University Press, Cambridge

Hollis, J.P. 1951. Bacteria in healthy potato tissue. *Phytopathology* 41:350-366

Holt, J.G. 1994. Bergey's Manual of Determinative Bacteriology. 9th ed. In: Holt, J.G., Kerig, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (eds). Williams and Wilkins, Baltimore MD. USA.

Horsch, R.B. and King, J. 1983. A covert contaminant of cultured plant cells: elimination of a *Hyphomicrobium sp*. from cultures of *Datura innoxia* (Mill.). *Pl. Cell Tiss. Org. Cult*. 2:21-28

Horwitz, J.P., Chua, J., Curby, R.J., Tomson, A.J. and Fisher, B.E. 1964. Substrates for cytochemical demonstration of enzyme activity. *J. Med. Chem*. 7:574-576

Hooykaas-Slogteren G.M.S, Hooykaas, P.J. and Schilperoort, R.A. 1984. Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefacines*. *Nature* 311:763-764

Houwe, V.D. and Swennen, R. 2000. Characterization and control of bacterial contaminants in *in vitro* cultures of banana (*Musa* spp.). *Acta Hort*. 530:69-79

Hucker, C.J. and Conn, H.J. 1923. Methods of gram staining. N.Y.St. *Agric. Exp. Stn. Tech. Bull.* 4:129

Hugenholtz, P., Pitulle, C., Hershberger, K.L. and Pace, N.R. 1998. Novel division level bacterial diversity in a Yellowstone. *Hot Spring. J. Bacteriol*. 180:366-376

Hugenholtz, P., Tyson, G.W., Webb, R.I., Wagner, A.M. and Blackall, L.L. 2001. Investigation of candidate division TM7, a recently recognized major lineage of the domain bacteria with no known pure-culture representatives. *Appl. Environ. Microbiol*. 67:411-419

Hussey, R.S. and Rancodori, R.W. 1982. Vesicular arbuscular mycorrhizae may limit nematode activity and improve plant growth. *P1. Dis*. 66:9-14

Hurek, T., Reihold-Hurek, B., Monlages, V.M. and Kellenberger, E. 1991. Infection of intact roots of Kallar grass and rice seedlings by *Azoarcus*. *Dev*. *Plant Soil Sci*. 48:235-247

Jacobs, M.J., Bugbee, W.M. and Gabrielson, D.A. 1985. Enumeration, location and characterization of endophytic bacteria within sugar beet roots. *Can. J. Bot*. 63:1262-1265

Jaizme-Vega, M.C. and Azcon, R. 1991. Effect of vesicular arbuscular mycorrhizal fungi on pineapple (*Ananas comosus* L. Merr.) in the canary Islands. *Fruits* 46:47-50

Jaizme-vega, M.C. and Azcon, R. 1995. Responses of some tropical and subtropical cultures to endomycorrhizal fungi. *Mycorrhiza* 5:213-217

Jaizme-vega, M.C., Delamo, M.E., Domingaez, P.T. and Romero, A.S.R. 2002. Effects of mycorrhization on the development of two cultivars of micropropagated banana. *Infomusa* 11:25-28

James, E.K. and Olivares, F.L. 1997. Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. *Crit. Rev. Pl. Sci*. 17:77-119

Jamal, A., Ayub, N., Usman, M. and Khan, A.G. 2002. Arbuscular mycorrhizal fungi enhance zinc and nickel uptake from contaminated soil by soybean and lentil. *Int. J. Phytoremed* 4 (3):203-221

Jarstfer, A.G. and Sylvia, D.M. 1994. Aeroponic culture of VAM fungi. In:Varma, A.K. and Hock, B. (eds.), *Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology*. Springer-Verlag, Berlin, pp. 427-441

Jeffries, P., Gianinazzi, S., Perotto, S., Turnao, K. and Barea, J.M. 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol. Fertility soil* 37:1-16

Jha, T.B. 1988. *In vitro* morphogenesis in cashew nut (*Anacardium occidentale* L.). *Indian J. Exp. Biol*. 26:505-507

Joseph, P.J. 1997. Management of rhizome rot of ginger using VA mycorrhizae and antagonists. Ph. D. Thesis Kerala Agricultural University, Thrissur, 192 p.

Kamoun, R., Lepoivre, P. and Boxus, P. 1998. Evidence for the occurence of endophytic prokaryotic contaminants in micropropagated plantlets of *Prunus cerasus* cv. Montmorency. *Pl. Cell Tiss. Org. Cult*. 52:57-59

Karmarkar, S.H., Keshavachandran, R., Nazeem, P.A. and Girija, D. 2001b. Hairy root induction in Adapathiyan (*Holostemma ada-kodien* K. Schum.). *J. trop. Agric.* 39:102-107

Karp, A. 1991. *Oxford Surveys Plant Molecular and Cell Biology*, volume 7. Oxford University Press, Oxford, pp. 1-58.

Kavitha, K. 2001. Management of damping off and improvement of growth in chilli (*Capsicum annuum* L.) with native species of arbuscular mycorrhizae and *Azospirillum*. M.Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 79p.

Kaya, C., Higgr, D., Kirnak, H. and Tas, 1. 2003. Mycorrhizal colonization improves fruit yield and water use efficiency in water melon (*Citrullus lanatus* Thumb) grown under well-watered and water-stressed condition. *P1. Soil* 253:2287-292

Keshavachandran, R. 1996. Morphogenic potential of shoot buds in cashew (*Anacardium occidentale* L.). In: *Developments in Plantation Crop Research*. pp. 34-39.

Keshavachandran, R. 2004. Report submitted to the DBT., New Delhi.

Keshavachandran, R. and Khader, A.M.D. 1990. Micropropagation of cashew (*Anacardium occidentale* L.). *J. Plantn. crops* 20:299-300

Keshavachandran, R. and Menon, R. 1997. Control and estimation of phenolic exudation in *in vitro* culture of cashew (*Anacardium occidentale* L.). National Symposium on Emerging trends in Plant Tissue Culture and Molecular Biology, Hyderabad. p. 37.

Keshavachandran, R. and Shelja, P.B. 1999. Genotypic difference in shoot bud culture of cashew (*Anacardium occidentale* L.). In: *Proceedings of Eleventh Kerala Science Congress*, Kasaragod, pp. 234-235.

Keshavachandran, R. and Riji, V.S. 2005. *Standardisation of in vitro micrografting techniques in cashew*. In: *Proceedings of National Symposium of Biotechnological Interventions for Improvement of Horticultural crops: Issues and Strategies*, Kerala Agricultural University. p. 205.

Khaled, L.B., Gomez, A.M., Ourraqi, E.M. and Oihabi, A. 2003. Physiological and biochemical responses to salt stress of mycorrhized and/or nodulated clover seedlings (*Trifolium alexandrinum* L.) *Agronomie* 23:571-580

Khaliq, A., Gupta, M.L. and Kumar, S. 2001. The effect of VAM fungus on growth of peppermint. *Indian Phytopath*. 54:82-84

Kiernan, J.M., Hendrix, J.W., Stoltz, L.P. and Marsonek, D.M. 1984. Characterization of strawberry plants produced by tissue culture and infected with specific mycorrhizal fungi. *Hort. Sci*. 19:883-885

Kimpinski, J. and Stur'z, A.U. 1996. Population growth of a rhadited nematode on plant growth promoting bacteria from potato tubers and rhizosphere soil. *J. Nematol.* 28:682-686

Kloepper, J.W., Ryu, C. and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Am. Phytopathology* 91:1259-1266

Kloepper, J.W., Schippers, B., and Bakkers, P.A.H.M. 1992. Proposed emanation of the term endorhizosphere. *Phytopathology* 82:726-727

Kloepper, J.W., Weig and Tuzun, S. 1992a. Rhizosphere population dynamics and internal colonization of cucumber by plant growth promoting Rhizobacteria which reduce systemic resistance to *Colletotrichum orbiculare*. *J. Biological Control Pl. Dis.* p. 185-191

Koike, Y., Hoshino, Y., Mii, M. and Nakano, M. 2003. Horticultural characterization of *Angelonia salicarifolia* plants transformed with wild type strains of *Agrobacterium rhizogenes*. *Pl. Cell Rep.* 21:981-987

Kouider, M., Skirvin, R.M., Korban, S.S., Widholm, J.M. and Hauptman, R. 1984. Adventitious shoot formation from red delicious apple cotyledons *in vitro*. *J. Hortic. Sci.* 59:295-302

Krishna, K.R. and Bagyaraj, D.J. 1983. Interaction between *Glomus fasiculatum* and *Sclerotium rolfsii* in peanut. *Can. J. Bot*. 61:2349-2351

Krishna, K.R. and Bagyaraj, D.J. 1986. Phenolics of mycorrhizal and uninfeted groundnut var. MGS-7. *Curr. Res.* 15:51-52

Kumar, S., Bhatt, V., Bhat, B.V. and Gupta, M.G. 2002. *Agrobacterium*-mediated transformation of Lucerne (*Medicago sativa* Linn.): Optimising Biological and Physical Parameters. *Indian J. Biotech.* 21:298-300

Kumari, S.M.P. and Balasubramanian, A. 1993. Effect of combined inoculation of VAM and *Azospirillum* on the growth and nutrient uptake of coffee seedlings. *Indian Coffee* 67:5-11

Lad., B.L., Chvan, S.S. and Madgum, M.B. 1994. Proceedings of the 2nd Asia Pacific International Conference Agricultural Biotechnology, Madras, March 6-10, p. 215.

Lakshmi-Sita, G. 1989. Differentiation of embryos and leafy shoots from callus cultures of cashew (*Anacardium occidentale* L.). In: *Abstracts XIII Plant Tissue Culture Conference*, 18-20 Oct., Shillong, India. Abstract No. 71.

Lamb, T.G., Tonkyn, D.W. and Kluepfel, D.A. 1996. Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. *Can. J. Microbiol.* 42:1112-1120

Lata, H., Andrade, D.Z., Sachaneberg, B., Bedir, E., Khan, L. and Moraes, R. 2003. Arbuscular mycorrhizal inoculation enhances survival rates and growth of micropropagated plantlets of *Echinacea pallid*. *Planta medica* 69:679-682

Lee, N. Wetzstein, H.V. and Sommer, H.E. 1985. Effect of quatum flux density on photosynthesis and chloroplast ultrastructure in tissue cultural plantlets. *PI. Physiol.* 78:637-641

Leifert, C. and Cassells, A.C. 2001. Microbial hazards in plant tissue and cell cultures. *In Vitro Cell. Dev. Biol. Plant* 37:133-138

Leifert, C. and Waites, W.M. 1992. Bacterial growth in plant tissue culture media. *J. Appl. Bacteriol.* 72:460-466

Leifert, C. and Woodward, S. 1998. Laboratory contamination management: the requirement for microbiological quality assurance. *Pl. Cell Tiss. and Organ Cult*. 52:83-88

Leifert, C., Morri, C. and Waites, W.M. 1994. Ecology of microbial saprophytes and pathogens in tissue culture and field grown plants; Reasons of contamination problems *in vitro* CRC. *Critical Rev*. *Plant Sci*., 13:139-183

Leshem, B. 1983. Growth of carnation *in vitro*. Anatomical structure of abnormal plantlets and the effect of agar concentrations in the medium on their formation. *Ann. Bot*. 52:413-415

Leva, A.R. and Falcone, A.M. 1990. Propagation and Organogenesis *in vitro* of *Anacardium occidentale* L. *Acta Horti*. 280:143-156

Leyns, F., Lambart, B., Joos, H. and Swing, J. 1990. Antifungal bacteria from different crops. In: Harnby (ed.), *Biological control of soil borne plant pathogens* D. p. 437-444.

Leyval, C. and Berthelin, J. 1989. Influence of acid-producing *Agrobacterium* and *Laccaria laccata* on pine and beech growth nutrient uptake and exudation. *Agr. Ecosys. Environ*. 28:313-319

Li, X.L., George, E. and Marschner, H. 1991. Extension of the phosphorus depletion zone in VA mycorrhizal white clover in a calcareous soil. *Pl. Soil* 136:41-48

Li, M., and Leung, D.W.M. 2003. Root induction in radiate pine using *Agrobacterium rhizogenes*. *Electronic J. Biotechnol.* 3:254-270

Li, M., Liu, P. and Liu, R. 2002. Effect of AMF on micropropagated taro (*Colocasia esculenta*). *Acta Hort. Sinica* 29:451-453

Liao J.P., Lin, X.G., Cao, Z.H., Shi, Y.Q. and Wong, M.H. 2003. Interactions between arbuscular mycorrhizae and heavy metals under sand culture experiment. *Chemosphere* 50(6):847-853

Lievens, C., Pylyser, M. and Boxus, P.H. 1989. Clonal propagation of *Anacardium occidentale* by tissue culture. *Fruits* 44:553-557

Lin, M.L. and Staba, J.E. 1961. Peppermint and spearmint tissue culture. I. Callus formation and submerged culture. *Lloydia* 24:139-143

Lin, X. and Hao, W. 1988. Effects of VAM inoculation on growth of several kinds of plants. In: Mahadevan, A., Raman, N. and Natarajan, K. (eds.), *Mycorrhizae for green Asia*. University of Madras, India, pp. 231-232.

Lin, M., Xu,Y., Sun, Y., Hou, S., Wang, Z. and Li, S. 2005. Submitted to Biotechnology Research Institute, Chinese Academy of Agricultural Science, Beijing, China (Press)

Lins, G.M., De, L., Trindacle, A.V. and Rocha, H.S. 2003. Utilization of *Gigaspora margarita* in micropropagated banana plants in different stages of rooting. *Revista Brasileira de Fruitcultura* 25(1):143-147

Linsmaier, E.M. and Skoog F. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant*. 18:100-127

Litz., R.E. and Conover, R.A. 1981. Effect of sex type and other factors on *in vitro* establishment and culture of *Carica papaya* L. explants. *J. Am. Soc. Horti. Sci*. 106:792-794

Liu, A., Hamel, C., Emi, A., Costa, C., Ma, B. and Smith, D.L. 2002. Concentrations of K, Ca and Mg in maize colonized by arbuscular mycorrhizal fungus under field conditions. *Can. J. Soil Sci.* 82:271-275

Lloyd, G. and McCown, B.H. 1980. Commercial feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip cultures. *Proc. Int. Plant Prop. Soc.* 30:421-427

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M. and Lelie, V.D.D. 2002. Endophytic bacteria and their potential applications. *Crit. Rev. Pl. Sci.* 21:583-606

Lopez, A., Pinochet, J., Fernandez, C., Clavert, C. and Camprubi, A. 1997. Growth response of OHF 333 pear root stock to arbuscular mycorrhizal fungi, phosphorus nutrition and Pratylenchus values infection. *Fundament. Appl. Nematol*. 20:87-93

Lovato, P.E., Gianinazzi-Pearson, V., Trouvelot, A. and Gianinazzi, S. 1996. The state of the art of mycorrhizas and micropropagation. *Adv. Hort. Sci.* 10:46-52

Lubraco, G., Schubert, A. and Previate, A. 2000. Micropropagation and mycorrhization of *Allium sativum*. *Acta Hort*. 530:339-343

Ludvig-Muller, J. 2000. Indole-3-butyric acid in plant growth and development. *Pl. Growth Reg.* 32:219-230

MacRae, S. and Staden, V.J. 1993. *Agrobacterium rhizogenes* mediated transformation to improve rooting ability of eucalyptus. *Tree Physiol*. 12:411-418

Mahadevan, A. 1970. Triggering mechanism in host-parasite interaction. *J. Sci. Ind. Res.* 29:23-36

Mahaffee, W.F. and Kloepper, J.W. 1997. Microbial changes in the bacterial communities of soil rhizosphere and endorhize. *Microbiol. Ecol*. 34:210-223

Malabadi, R.B. and Nataraja, K. 2003. Alkaloid biosynthesis influenced by *Agrobacterium rhizogenes* mediated-transformation and bioreactor in *Clitoria ternatea* (Linn.). *Pl. Cell Biotech. mol. Biol.* 4:169-178

Malick, C.P. and Singh, M.B. 1980. In: Plant Enzymology and Histo Enzymology. Kalyani Publishers, New Delhi, p. 286.

Mandel, M. and Higa, A. 1970. Calcium dependent bacteriophage DNA infection. *J. Mol. Biol.* 53:159-162

Mantell, S.H., Boggetti, B., Bessa, A.M.S., Lemos, E.P., Abdelhadi, A. and Mneney, E.E. 1998. Micropropagation and micrografting methods suitable for international transfers of cashew. *Proceedings of International Cashew and Coconut Conference* 1997; Dar es Salaam, Tanzania, pp 95-107.

Marin, M., Mari, A., Ibarra, M. and Garcia-Ferriz, L. 2003. Arbuscular mycorrhizal inoculation of micropropagated persimmon. *J. Hort. Sci. and Biotechnol.* 78(5):734-738

Marin-Zamora, M., Garcia-Ferriz, L., Ghorbel, R., Ybarra, M. and Mari, A. 2002. Effects of arbuscular mycorrhizae inoculation on micropropagated olive plants. *Acta Hort*. 586:907-910

Martin, J.A., Gella, R. and Herrera, M.C. 1988. Stomatal structure and functioning as a response to environmental changes in acclimalized micropropagated *Prunus cerasus* L. *Ann. Bot*. 62:663-670

Marx, D.H. 1975. Mycorrhizae and the establishment of trees on strip-mined land, Ohio. *J. Sci*. 75:88-297

Mathur, N. and Vyas, A. 1999. Improved biomass production, nutrient uptake and establishment of *in vitro* raised *Zyzyphus mauritiana* by VA mycorrhiza. *J. Pl. Physiol*. 155:129-132

Mauch, F., Mauch-Mani, B. and Boller, T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanases. *Pl.Physiol*. 88:936-942

McAfee, B.J., White, E.E., Pelches, L.E. and Lapp, M.S. 1993. Root induction in pine (*Pinus*) and larch (*Larix*) spp. Using *Agrobacterium rhizogenes*. *Pl. Cell, Tiss. and Organ Cult*. 34:53-62

McDonald, R.M. 1981. Routine production of axenic vesicular-arbuscular mycorrhizas. *New Phytol*. 89:87-93

Mehta, V. and Ram, H.Y. 1980. Regeneration of plantlets from cotyledons of *Cajanus cajan*. *Indian J. Exp. Biol.* 18:800-802

Mclnroy, J.A. and Kloepper, J.W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Pl. Soil* 173:337-342

Miche, L. and Balandreau, J. 2001. Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. *Appl. Environ. Microbiol.* 67:3046-3052

Mihaljevic, S., Katavic, V. and Jelaska, S. 1999. Root formation in micropropagated shoots of *Sequoia sempervirens* using *Agrobacterium*. *Pl. Sci.* 141:73-80

Mihaljevic, S., Stipkovic, S. and Jelaska, S. 1996. Increase of root induction in *Pinus nigra* explants using agrobacteria. *Pl. Cell Rep.* 15:610-614

Miller, R.M. and Jastrow, J.D. 1990. Hierarchy of root and mycorrhizal fungal interaction with soil aggregation soil. *Biol. Biochem*. 22:579-584

Mneney, E.E. and Mantell, S.H. 2002. Clonal propagation of cashew (*Anacardium occidentale* L.) by tissue culture. *J. Hort. Sci. and Biotech.* 77(6):649-657

Morton, J.B. and Benny, G.L. 1990. Revised classification of arbuscular mycorrhizal fungi. (zygomycetes): a new order Glomales, two new suborders, Glominae and Gigaasporinae and two new families,

Acaulosporaceae and Gigasporaceae with an emendation of Glomaceae. *Mycotaxon* 37:471-491

Mosse, B. 1962. *The establishment of Mycorrhizal Infection under Aseptic Conditions*. Experimental Station Report for 1961, Rothamsted, 80p.

Mosse. B. 1973 Advances in the study of vesicular arbuscular mycorrhiza. *A. Rev. Phytopath* . 11:171-196

Mosse, B. and Hepper, C.M. 1975. Vesicular arbuscular infection in root organ cultures. *Physiol. Pl. Path.* 5:215-225

Mosse, B. and Thompson, J.P. 1984. Vesicular-arbuscular endo mycorrhizal inoculum production, 1, Exploratory experiments with beans (*Phaseolus vulgaris*) in nutrient flow culture. *Can. J. Bot*. 62:1523-1530

Mullis, K.B. 1990. The unusual origins of the polymerase chain reaction. *Scient. Am*. 262:56-65

Mundt, J.O. and Hinkle, N.F. 1976. Bacteria within ovules and seeds. *Appl. Environ. Microbiol.* 32(5):694-698

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. 15:473-497

Nair, S.R. and Mohanakumaran, N. 1993. Standardisation of tissue/apical/meristem culture techniques in horticultural crops of Kerala, Final Technical Report (USDA). IN-AES-343, Thiruvananthapuram, Kerala, pp. 3-15.

Nagarajan, P., Radha, N.V., Kandasamy, D., Oblisami, G. and Jayaraj, S. 1989. Effect of combined inoculation of *Azospirillum brasilense* and *Glomus fasciculatum* on mulberry. *Madras agric. J.* 76:601-605

Nakasone, H.Y., Paull, R.E. 1998. *Tropical Fruits*. CAB international, U.K. p. 432.

Nambiar, M.C. and Iyer, R.D. 1988. Tissue culture in cashew: Potential, priorities and applications. In: Tata, S.N. (ed.), *Proceedings of the National Seminar on Plant Tissue Culture*, ICAR, New Delhi, pp. 149-155.

Nemec, S. and Meridith, F.F. 1981. VAM infection may change the biochemical composition of the host plant. Information on the effects of VAM on

histochemical and biochemical composition of host plant is limited. *Am. Bot*. 47:351

*Nickell, G.L. 1982. Kirk-othmer Encyclopaedia of chemical technology. New York, USA. pp. 321-322

Niedz, R.P. and Bausher, M.G. 2002. Control of *in vitro* contamination of explants from greenhouse and field-grown trees. *In Vitro Cell. Dev. Biol. Pl.* 38:468-471

Nilsson, O. and Olsson, O. 1997. Getting to the root: the role of *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. *Physiol. Plant*. 100:463-473

Ohler, J.G. 1979. Cashew. Koninklijk institaut voor de Tropen; Teskin, Zutphen Co. Amsterdam, pp. 18-19.

Palazon, J., Cusido, R.M., Roig, C. and Pinol, M.T. 1997. Effect of *rol* genes from *Agrobacterium rhizogenes* TL-DNA on nicotine production in tobacco root cultures. *Pl. Physiol. Biochem*. 35:155-162

Pandey, P.K. and Goswami, B.K. 2005. *Bacillus subtilis*: An ecofriendly and effective antagonist of *Fusarium udum* causing Pigeonpea wilt. *Proc. Nat. Acad. Sci. India* 75(B), IV

Panicker, B., Thomas, P., Janakiran, T., Venugopalan, R. and Sathyanarayana, B.N. 2007. Influence of cytokininlevels on *in vitro* propagation of shy suckering chrysanthemum 'Arka Swarna' and activation of endophytic bacteria. *In Vitro Cell. Dev. Biol. Pl.* 43:614-622

Patriquin, D.G. and Doberinner, J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in endorhizosphere of maize and other grasses in Brazil. *Can. J. Microbiol.* 24:734-747

Patena, L., Sutu, E.G. and Dandekar, A.M. 1988. Root induction by *Agrobacterium rhizogenes* in a difficult to root woody sp. *Acta* 227: 324-329

Pawlicki, N., Sanywan, R.S. and Norreel, B.S. 1992. Factors influencing the *Agrobacterium tumefaciens*-mediated transformation of carrot (*Daucus carota* L.). *Pl. Cell Rep.* 31:129-139

Petit, A., Berkaloff, J. and Tempe, J. 1986. Multiple transformation of plants cells by *Agrobacterium* may be responsible for the complex organization of hairy root T-DNA. *Mol. Gen. Genetic* 202:388-393

Pfleger, F.L., Stewart, E.L. and Noyd, R.K. 1994. Role of VAM fungi in mine land revegetation. In: Pfleyar. F.L. and Linderman P.G. (eds.), *Mycorrhizae and Plant Health* APS, Minnesota, USA, pp. 47-81

Philip, V.J. 1984. *In vitro* organogenesis and plantlet formation in cashew (*Anacardium occidentale* L.). *Ann. Bot*. 54:149-152

Phillips, J.M. and Hayman, D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycological Soc.* 55:158-161

Philip, V.J. and Unni, P.N. 1984. *In vitro* propagation of cashew for crop improvement. In: Bhaskara Rao, E.V.V. and Khan, H.H. (eds), *Cashew Research and Development*. CPCRI, Kasaragod, p. 78-82.

Pierik, R.L.M. 1987. *In vitro* culture of higher plants as a tool in the propagation of horticultural crops. *Acta Hort*. 226:25-40

Pierik, R.L.M. 1990. Rejuvenation and micropropagation. In: Nijikanp, H.J.J.L. (ed.), *Progress in Plant Cellular and Molecular Biology*. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 91-101.

Posada, F. and Vega, F.E. 2005. Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in Cocoa seedlings (*Theobroma cocao*). *Mycologia* 97:1195-1200

Potrykus, I. 1990. Gene transfer to plants: assessment and perspectives. *Physiol. Plant*. 79:206-210

Preece, J.E. and Compton, M.E. 1991. Problems with explant exudation in micropropagation. In: Bajaj, Y.P.S. (ed.), *Biotechnology in Agriculture and Forestry*, Vol. 17. Springer - Verlag, Berlin, Heidelberg, New York, p. 168-189.

Preece, J.E. and Sutter, E.G. 1991. Acclimatization of micropropagated plants to the green house and field. In: Debergh, P.C. and Zimmerman, R.H. (eds.),

Micropropagation:Technology and Applications. Academic Rublishers, Dordercht, Netherlands, pp. 71-93.

Quadt-Hallman, A., Benhamou, N. and Kloepper, J.W. 1997. Bacterial endophytes in cotton mechanisms of entering the plant. *Can. J. Microbiol.* 43:557-582

Quadt-Hallman, A. and Kloepper, J.W. 1996. Immunological detection and localization of the cotton endophyte *Enterobacter asburiae* JM22 in different plant species. *Can. J. Microbiol.* 42:1144-1154

Radeva, G. and Selenska-Pobell, S. 2005. Bacterial diversity in water samples from uranium wastes as demonstrated by 16S rDNA and ribosomal intergenic spacer amplification retrievals. *Can. J. Microbiol.* 51:910-923

Raj Bhansali, R. 1990. Somatic embryogenesis and regeneration of plantlets in pomegranate. *Ann. Bot*. 66:1249-1253

Raju, P.S., Clark, R.B., Ellis, J.R. and Maranville, J.M. 1990. Effect of species of VA-mycorrhizal fungi on growth and mineral uptake of sorghum at different temperatures. *Pl. Soil* 121(2):165-170

Raman, N. and Sambandan, K. 2000. Axenic germination of *Scutellospora erythropa* and *Scutellospora migra in vitro* conditions. *Indian J. Exp. Biol*. 38:1159-1163

Raman, N., Sahadevan, C. and Srinivasan, V. 2001. Growth of AMF on *in vitro* root organ culture of *Sorghum vulgare* and *Saccharum officinarum*. *Indian J. Exp. Biol*. 39:1293-1298

Ramanayake, S.M.S.D. and Kovoor, A. 1997. *In vitro* micrografting of cashew. *Proceedings of XIII Plant Tissue Culture Conference*, Oct 18-20, Shillong, p.86

Ramesh, B. 1990. *Ex vitro* establishment of jack plantlets (*Artocarpus caryophyllus*). M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 52p.

Rao, V.U. and Rao, A.S. 1998. Interactive effect of VAM fungi and *Aspergillus niger* on nutrient and biochemical constituents in two grin legumes. *Int. J. Trop. Agric*. 14:115-121

Rao A.V. and Tak, R. 2002. Growth of different tree species and their nutrient uptake in limestone mine spoil as influenced by arbuscular mycorrhizal (AM) fungi in Indian arid zone. *J. Arid Environ*. 51(1):113-123

Rapparini, F., Baraldi, R., Bertazza, G., Brazanti, B. and Predieri, S. 1994. Vesicular-arbuscular mycorrhizal inoculation of micropropagated fruit trees. *J. Hort. Sci.* 69:1101-1109

Ravenschlag, K., Sahm, K., Knoblauch, C., Jorgersen, B.B. and Aman, R. 2000. Community structure, cellular rRNA content and activity of sulfate-reducing bacteria in marine arctic sediments. *Appl. Environ. Microbiol.* 66:3592-3602

*Reuther, S. 1986. Characteristics of plant produced in tissue culture and their effects on futher cultures. *Zier Pflan* 16:172-174

Reysenbach, A.L., Longnecker, K. and Kirshtein, J. 2000. Novel bacterial and archaeal lineages from an *in situ* growth chamber deployed at a mid-atlantic ridge hydrothermal vent. *Appl. Environ. Microbiol.* 66:3798-3806

Rhoder, L.H. 1980. The use of mycorrhizae in crop production system-*Outl. Agric* 10:275-281

Rhodes, M.J.C., Robins, R.J., Lindasy, E., Arid, M., Payne, J., Pare, A.J. and Walton, N.J. 1989. *Primary and Secondary Metabolism of Plant Cell Cultures*. Springer-Verlag, New York, pp. 58-73

Ricker, A. J. 1930. Studies on infections hairy roots of necessary apple trees. *J. Agric. Res.* 41:438-446

Rillig, M.C. and Steinberg, P.D. 2002. Glomalin production by an arbuscular mycorrhizal fungus: a mechanism of habit modification. *Soil Biol. Biochem*. 34: 1371-1374

Rizzardi, V. 1990. Effect of inoculation with VA mycorrhizal fungi on the growth of micropropagated *Musa acuminata* clone *Grand Naine*. *Revista di Agricoltura Subtropicale e tropicale* 84:483-484

Robert, C.P. 1998. Management of foot rot of black pepper (*Piper nigrum* L.) with vesicular arbuscular mycorrhizae and antagonists. Ph. D. thesis, Kerala Agricultural University, Thrissur, 227p.

Rosenblueth, M. and Martinez-Romero, E. 2006. Bacterial endophytes and their interactions with hosts. *Mol. Plant Microbe. Interact*. 19:827-837

Rubos, A.C. and Pryke, J.A. 1984. Morphogenesis in embryogenie tissues of apple. *J. Hort. Sci.* 59:469-475

Rufykin, G., Thirty, Y., Wang, L., Delvaux, B. and Decleck, S. 2002. Uranium uptake and translocaiton by the arbuscular fungus, *Glomus intrardices*, under root organ culture conditions. *New Phytol*. 156:275-281

Ruiz-Lozano, J.M., Azcon, R. and Comez, M. 1996. Alleviation of salt stress by arbuscular mycorrhizal *Glomus* species in *Lactuca sativa* plants. *Physiol. P1ant*. 98:767 -772

Safir, G.R., Boyer, J.S. and Gerdemann, J.W. 1971. Mycorrhizal enhancement of water transport in soybean. *Science* 172:581-583

Safir, G.R., Boyer, J.S. and Gerdemann, J.W. 1972. Nutrient status and mycorrhizal enhancement of water transport in soybean. *PI. Physiol.* 49:700-703

Salih, S., Waterworth, H. and Thompson, D.A. 2001. Role of plant tissue cultures in international exchange and quarantine of germplasm in the United States and Canada. *HortScience* 36:1015-1021

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Second ed. Cold Spring Harbor Laboratory Press, New York, USA, 1322p.

Sanchez–Blanco, M.J., Ferrandez, T., Morales, M.A., Morte, A. and Alarcon, J.J. 2004. Variations in water stratus, gas exchange and growth in *Rosmarinus officinalis* plants infected with *Glomus deserticola* under drought conditions *J. Pl. Physiol.*_161:675-682

Sardinha, R.M.A., Bessa, A.M.S., Blake, J., Guyyer, D., Cassami, C. and Tamba-Bungue, P. 1993. Selection of superior genotypes of cashew (*Anacardium occidentale* L.) in Guinea-Bissau and the development of *in vitro* technique for their propagation. Final Summary Rept., Contract TS-2-A-01, Lisbon, 67p.

Sangwan, R.S., Ducrocq, C. and Sangwan-Norreel, B.S. 1991. Effect of culture conditions on *Agrobacterium* mediated transformation of *Datura*. *Pl. Cell Rep.* 10:90-93

Sarmento, G.G., Alpert, K., Tang, F.A. and Punja, Z.K. 1992. Factors influencing *Agrobacterium tumefaciens* mediated transformation and expression of kanamycin resistance in pickling cucumber. *Pl. Cell Tiss. Org. Cult*. 31:185-193

Sbrana, C., Giovannetti, M. and Vitagliano, C. 1994. The effect of mycorrhizal infection on survival and growth renewal of micropropagated fruit rootstocks. *Mycorrhiza* 5:153-156

Schalk, J.M., Peterson, J.K. and Hamalle, R.J. 1987. The abdominal flora of the banded cucumber beetle (*Diabrotica balteata* Leconte). *J. Agric. Entomol.* 4:333-336

Schellenbaum, L., Berta, G., Ravolanirisa, F., Tisserant, B., Gianinazzi, S. and Fitter, A. H. 1991. Influence of endomycorrhizal infection on root morphology in a micro propagated woody plant species *Vitis vinifera* (L.). *Ann. Bot*. 68:135-234

Schleif, R.F. and Wensink, P.C. 1981. Practical Methods in Molecular Biology, Springer-Verlag.

Schmulling, T., Schell, J. and Spena, A. 1988. Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO J*. 7:2621-2629

Schubert, A., Mazzitelli, M., Ariusso, O. and Eynard, I. 1990. Effects of vesicular-arbuscular mycorrhizal fungi on micropropagated grape vines: Influence of endophytic strain, P fertilization and growth medium. *Vitis* 29:5-13

Schulz, B. and Boyle, C. 2006. In: Schulz, B.J.E., Boyle, C.J.C. and Sieber, T.N. (eds.), *What are endophytes ? Microbial Root Endophytes*. Springer-Verlag, Berlin, pp. 1-13.

Scott, R.I., Chard, J.M., Lennard, J.H. and Graham, D.C. 1996. Penetration of potato tuber lenticels by bacteria in relation to biological control of blackleg disease. *Potato Res.* 39:333-344

Selvaraj, T. and Subramanian, G. 1990. Phenols and lipids in mycorrhizal and non-mycorrhizal roots of *Sesamum indicum*. *Curr. Sci.* 59:471-473

Severn-Ellis, A. 1999. Microorganisms may improve growth of micropropagated banana plants. *Neltropika Bull.* 303:35-36

Sevon, N. and Oksman-Caldentey, K.M. 2002. *Agrobacterium rhizogenes* mediated transformation: Root cultures as source of alkaloids. *Planta Med.* 68:859-868

Sharma, K.K. and Bhojwani, S.S. 1990. Histological aspects of *in vitro* root and shoot differentiation from cotyledon explants of *Brassica juncea* (L.) Czern. *Plant Sci.* 69:207-214

Shashikara, B.N., Reddy, B.J.D. and Bagyaraj, D.J. 1999. Response of micropropagated banana plantlets to *Glomus mosseae* at varied levels of fertilizer phosphorus. *Indian J. Exp. Biol.* 37:499-502

Shen, W.H., Petit, A., Guern, J. and Tempe, J. 1988. Hairy roots are more sensitive to auxin than normal roots. *Proc. Nat / Aca. Sci.* USA. 85:3417-3421

Simon, L., Lalonde, M. and Bruns, T.D. 1992a. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. *Appl. Environ. Microbiol.* 58:291-295

Simon, L., Levesque, R.C. and Lalonde, M. 1992b. Rapid quantitation by PCR of endomycorrhizal fungi colonizing roots. *PCR Methods Applic.* 2:76-80

Simon, L., Levesque, R.C. and Lalonde, M. 1993. Identification of endomycorrhizal fungi colonizing roots by fluorescent single-strand conformation polymorphism-polymerase chain reaction. *Appl. Environ. Microbiol.* 59:4211-4215

Simpson, R.B., Spielmann, A., Margossian, L. and Mcknight, T.A. 1986. Adisarmed binary vector from *Agrobacterium tumefaciens* function in *Agrobacterium rhizogenes*. *Plant Mol. Biol.* 6:403-415

Sindhu, S.S., Gupta, S.K. and Dadarwal, K.R. 1999. Antagonistic effect of *Pseudomonas* spp. on pathogenic fungi and enhancement of plant growth in green gram (*Vigna radiata*). *Biol. Fertil. Soils* 29:62-68

Sindhu, S.S., Suneja, S. and Dadarwal, K.R. 1999. Antagonistic effect of *Pseudomonas sp*. on pathogenic fungi and enhancement of plant growth in green gram. *Biol. Fertil. Soils*: 62-68

Singh, H.P., 1998. Keynote address. National seminar on Cashew Development in India: Challenges and Opportunities. Held at Goa on 1st and 2nd March.

Sivaprasad, P. and Sulochana, K.K. 2004. Integration of arbuscular mycorrhizal technology with micro-propagation. FIB, Govt. of Kerala, Thiruvananthapuram, 22p.

Sivaprasad, P., Joseph, P.J. and Balakrishnan, S. 2000. Management of foot rot of black pepper with arbuscular mycorrhizal fungi (AMF). In: Jain, R. K. (ed.), *Proceedings of International Conference on Integrated Plant Disease Management Sustainable Agriculture* Volume 1, 11-15 November, 1997; Indian Phytopathological Society, New Delhi, pp 341-342.

Sivaprasad, P., Ramesh, B., Mohanakumaran, N., Rajmohan, K. and Joseph, P.J. 1995a. VA mycorrhiza for the *ex vitro* establishment of tissue culture plantlets. In: Adholeya, A. and Singh, S. (eds.), *Mycorrhiza: Biofertilizer for the future*. Proceedings of an third national conference on Mycorrhizae, August 5-10, 1995; Tata Energy Research Institute, New Delhi, pp.281-283.

Sivaprasad, P., Suneetha, S., Joseph, P.J., Sulochana, K.K. and Rajmohan, K. 1999a. Influence of arbuscular mycorrhizal fungi (AMF) on establishment and growth of tissue culture plantlets of banana and alocasia. In: *Proceedings of the National Conference on Mycorrhiza: Association and distribution of mycorrhizal fungi under varying edapho climate conditions*. March 5-7, Bhopal, India. Abstr. 72

Sivaprasad, P., Suneetha, S., Joseph, P.J., Sulochana, K.K. and Rajmohan, K. 1999b. Effect of inoculation with vesicular-arbuscular mycorrhizal fungi on the growth of micropropagated *Musa accuminata* clone 'Grand Nain'. *Rivista di Agricolture Subtropicale e Tropicale* 84: 473-484

Sivaprasad, P and Rai, P.V. 1985. Photosyntheis and competition for photosynthates in tripartite symbiosis. *Curr. Sci.* 54:468-469

Spano, L., Mariotti, D., Cardarelli, M., Branca, C. and Costantino, P. 1988. Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. *Pl. Physiol.* 87:479-483

Smith, S.E. and Read D.J. 1997. *Mycorrhizal symbiosis*. Academic Press, Inc San Diego California, 342p.

Sreelatha, V. 1992. Improvement of propagation efficiency of *Anthurium* spp. *in vitro*. Ph.D. thesis, Kerala Agricultural University, Thrissur, 119p.

St. John, T.V., Hays, R.I. and Reid, C.P.P. 1981. A new method for producing pure vesicular-arbuscular mycorrhiza-host cultures without specialized media. *New Phytol.* 89:81-86

Stachel, S.E., Nester, E.W. and Zambryski, P.C. 1986. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proc. Natl. Acad. Sci.* USA. 83(2):379-383

Stackebrandt, E., Wilt, D., Kemmerling, C., Kroppenstedt, R.M. and Liesack, W. 1991. Designation of streptomycete 16S and 23S rRNA based target regions for oligonucleotide probes. *Appl. Environ. Microbiol*. 57:1468-1477

Stead, D.E., Elphinstone, J.G., Weller, S., Smith, N. and Hennessy, J. 2000. Modern methods for characterizing, identifying and detecting bacteria associated with plants. *Acta Hotic*. 530:45-57

Stein, L.Y., Jones, G., Alexander, B., Elmund, K., Wright -Jones, C. and Nealson, K. H. 2002. Intriguing microbial diversity associated with metal rich particles from freshwater reservoir. FEMS *Microbiol. Ecol*. 42:431-440

Steinberg, P.D. and Rillig, M.C. 2003. Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. *Soil Biol. Biochem*. 35:191-194

Strobel, G.A. and Nachmias, A. 1985. *Agrobacterium rhizogenes* promotes the initial growth of base root stock almound. *J. Gen. Microbiol*. 131:1245-1249

Strobel, G.A., Nachnian, A. and Heswm. 1988. Improvement in the growth and yield of olive trees by transformation with Ri plasmid of *Agrobacterium rhizogenes*. *Can. J. Bot*. 66:2581-2585

Strobel, G., Daisy, B., Castillo, U. and Harper, J. 2004. Natural products from endophytic microorganisms. *J. Nat. Pro.* 67:257-268

*Strullu, D.G. and Gerault, A. 1977. Etude des ectomycorhizes a Basidiomycete et a Ascomycete de Betula pubescens en microscopie electronique. *Comptes rendus Academic des Sciences Paris* 284:2243-2246

*Strullu, D.G. and Gourret, J.P. 1980. Donnees ultrastructurales sur l'integration cellulaire de quelques parasites ou symbiotes de plantes. II. Champignons mycorhiziens. *Bulletin Societe Botanique France* 127:97-106

Subramanian, K.S., Charest, C., Dwyer, L.M. and Hamilton, R.I. 1995. Arbuscular mycorrhizal and water relations in maize under drought stress at tasselling. *New phytol.* 129:643-650

Suslow, T.V., Schroth, M.N. and Isaka, M. 1982. Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. *Phytopathology* 72:917-918

Sutter, E. 1988 Morphological, physical and chemical characteristics of epicuticular wax on ornamental plant regenerated *in vitro*. *Ann. Bot*. 55:321-329

Sy, M.O., Martinelli, L. and Scienza, A. 1991. *In vitro* organogenesis and regeneration in cashew (*Anacardium occidentale* L.). *Acta Hort*. 289:267-268

Tanprasert, P. and Reed, B.M. 1997. Detection and identification of bacterial contaminants from strawberry runner explants. *In vitro Cell. Dev. Biol. Pl.* 33:221-226

Taylor, J. and Harrier, L.A. 2001. A comparison of development and mineral nutrition of micropropagated Fragaria X ananassa cv. Elvira (strawberry) when colonized by nine species of arbuscular mycorrhizal fungi. *Appl. Soil Ecol*. 18(3):205-215

Tepfer, D. 1984. Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. *Cell* 37:959-967

Thanuja, T.V., Hegde, R.V. and Sreenivasa, M.N. 2002. Induction of rooting and root growth in black pepper cuttings (*Piper nigrum* L.) with arbuscular mycorrhiza indigenous to Mexico. *Scientia Horticulturae* 92:347-359

Thimmappaiah. 1997. Ph.D. thesis, Mangalore University, India, 264p.

Thimmappaiah, Anil, S.R. and Iyer, R.D. 2001. Cashew. In: Parthasarathy, V., Bose, T. K. and Das, P. (eds.), *Biotechnology of Horticultural Crops*, Vol. 2. Naya Prakash, Calcutta, pp. 609-629.

Thimmappaiah and Shirly, R.S. 1996. Micropropagation studies in cashew (*Anacardium occidentale* L.). *Natl. Symp. on Hort. Biotech*. (souvenir), Bangalore, p.65.

Thimmappaiah and Shirly, R.S 1999. *In vitro* regeneration of cashew (*Anacardium occidentale* L.). *Indian J. Exp. Biol*. 37:384-390

Thimmappaiah, Shirly, R.S. and Sadhana, P.H. 2002. *In vitro* propagation of cashew from young trees. *In Vitro Cell Dev. Biol. Plant* 38:152-156

Thomas, P. 2004a. *In vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause for degeneration of long-term micropropagated triploid watermelon cultures. *Pl. Cell Tiss. Org. Cult*. 77:173-179

Thomas, P., Kumari, S., Swarna, G.K. and Gowda, T.K.S. 2007a. Papaya shoot tip associated endophytic bacteria isolated from *in vitro* cultures and host-endophytic interaction *in vitro* and *in vivo*. *Can. J. Microbiol.* 53:380-390

Thomas, P., Kumari, S., Swarna, G.K., Prakash, D.P. and Dinesh, M.R. 2007b Ubiquitous presence of fastidious endophytic bacteria in field shoots and index-negative apparently clean shoot-tip cultures of papaya. *Pl. Cell Rep.* 26:1491–1499

Thomas, P., Swarna, G.K., Patil, P. and Rawal, R.D. 2008a. Ubiquitous presence of normally non-cultivable endophytic bacteria in field shoot tips of banana and their gradual activation of quiescent cultivable form in tissue cultures. *Pl. Cell Tiss. Org. Cult*. 93:39-54

Thomas, P. and Prakash, G.S. 2004. Sanitizing long-term micropropagated grapes from covert and endophytic bacteria and preliminary field testing of plants after eight years *in vitro*. *In Vitro Cell. Dev. Biol. Pl.* 40:603–607

Thomas, P., Swarna, G.K., Roy, P.K. and Patil, P. 2008. Identification of culturable and originally non-culturable endophytic bacteria isolated from shoot tip cultures of banana cv. Grand Naine. *Pl. Cell Tiss. Org. Cult*. 93:55-63

Torrey, J.G. 1988. In: Hanover, J.W. and Kealthly, D.E. (eds.), *The genetic manipulation of woody plants*. Plenum Press, New York, London, pp. 1-21.

*Trindade, A.V., Lins, G.M.D. and Maia, I.C.S. 2003. Substrates and mycorrhizal fungus for micropropagation of banana seedlings in the acclimatization phase. *Revista Brasileira de Fruticultura* 25 (1):137-142

Ullmann, A., Jacob, F. and Monod, J. 1967. Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β galactosidase structural gene of *Escherichia coli*. *J. Mol.Biol*. 24:339-341

Ulrich, K., Stauber, T. and Ewald, D. 2008. *Paenibacillus* – a predominant endophytic bacterium colonizing tissue cultures of woody plants. *Pl. Cell Tiss. Organ Cult*. 93(3):347-351

Usami, S., Morikawa, S., Takebe, I. and Machida, Y. 1987. Absence in monocotyledonous plants of the diffusable plant factors inducing T-DNA circularization and *vir* gene expression in *Agrobacterium*. *Mol. Gen. Genet*. 209:221-226

Uosukainen, M. and Vestberg, M. 1994. Effect of inoculation with arbuscular mycorrhizas on rooting, weaning and subsequent growth of micropropagated *Malus* (L.) Moench. *Agric. Sciences Finlandia* 3:269-279

Vance, C.P. 1983. *Rhizobium* infection and nodulation: a beneficial plant disease ? *Annu. Rev. Microbiol.* 37:399-424

Varma, C.K.Y. 1995. Effect of *Azospirillum* inoculation on establishment and growth of bush pepper. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 87p.

Varma, A. and Schuepp, H. 1994. Infectivity and effectiveness of *Glomus intraradices* on micropropagated plants. *Mycorrhiza* 5:29-37

Venkatachalam, P., Jayashree, R., Rekha, K., Sushmakumari, S., Sobha, S., Jayasree, P.K., Kala, R.K. and Thulaseedharan, A. 2007. Rubber Tree (*Hevea brasiliensis* Muell. Arg). In: Wang, K. (ed.), *Methods in Molecular Biology volume 344: Agrobacterium protocols, volume 2*, Humana Press Inc, Totowa, pp. 153-164.

Vidal. M.T., Aguilar, C.A., Barea, J.M. and Pliego-Alfaro, F. 1992. Mycorrhizal inoculation enhances growth and development of micropropagated plants of avocado. *Hort. Sci.* 27:785-787

Viss, P.R., Brooks, E.M. and Driver, J.A. 1991. A simplified method for the control of bacterial contamination in woody plant tissue culture. *In Vitro Cell. Dev. Biol. Pl.* 27P: 42

Wang, C. and Dang, H. 2008. Incidence of ESBLs and plasmid-mediated AmpC beta-lactamase genes and integrons in environmental Enterobacteriaceae isolated from the eutrophic Jiaozhou Bay, China (Press).

Weisburg, W.G., Bars, S.M., Pelletier, D.A. and Lane, D.J. 1991. 16S Ribosomal DNA amplification for phylogenetic study. *J. Bact*. 173:697-703

White, F.F., Ghidossi, G., Gordon, M.P. and Nester, E.W. 1982. Tumour induction by *Agrobacterium rhizogenes* involves the transfer for plasmid DNA to the plant genome. *Proc. Natl. Acad. Sci. USA* 79:3193-1397

White, F.F., Taylor, B.H., Huffman, G.D., Gordon, M.P. and Nester, E.W. 1985. Molecular and genetic analysis of the transferred DNA regions of the root inducing plasmid of *Agrobacterium rhizogenes. J. Bact*. 164:33-44

Wilkins, C.P., Cabrera, J.L.P. and Dodds, J.H. 1985. Tissue culture propagation of trees. *Outlook on Agric*. 14(1):2-13

Willmitzer, L., Sanchez-Serrano, J., Buschfeld, E. and Schell, J. 1982. DNA from *Agrobacterium rhizogenes* is transferred to and expressed in axenic hairy root plant tissues. *Mol. Gen. Genet*. 186:16-22

Wilson, D. 1993. Induced mutagenesis in rose under *in vitro* and *ex vitro* culture. Ph. D. thesis, Kerala Agricultural University, Thrissur, 239p.

Woodroof, J.G. 1967. *Tree nuts: Production, Processing and Products*. Volume 1. AVI Publishing Company Incorporation, West port, CT, USA, pp. 221-225.

Wordragon, M.F. van, Ouwerkerk, P.B.F. and Dons, H.J.M. 1992. *Agrobacterium rhizogenes* mediated induction of apparently untransformed roots and callus in Chrysanthemum. *Pl. Cell Tiss. Org. Cult*. 30:149-157

Wyss, P. and Bonfante, P. 1993. Amplification of genomic DNA of arbuscular-mycorrhizal (AM) fungi by PCR using short arbitrary primers. *Mycol. Res.* 97:1351-1357

Yano-Melo, A.M., Saggin O.J. Jr., Lima-Filho, J.M., Melo, N.F. and Maia, L.C. 1999. Effect of arbuscular mycorrhizal fungus on the acclimatization of micropropagated banana plantlets. *Mycorrhiza* 9:119-123

Zdravkovic-Korac, S., Muhovski, Y., Druart, P., Calic, D. and Radojevic, L. 2004. *Agrobacterium rhizogenes* mediated DNA transfer to *Aesculus hippocastanum* L. and regeneration of transformed plants. *Pl. Cell Rep*. 22:698-704

*Zeleznik, A., Baricevic, D. and Vodnik, D. 2002. Micropropagation and acclimatization of yellow gentian, *Kmetijstvo 7*9(1):253-259

Zinniel, D.K., Lambrecht, P., Harris, N.B., Feng, Z., Kuczmarski, D., Higley, P., Ishimaru, C.A., Arunakumari, A., Barletta, R.G. and Vidaver, A.K. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol.* 68:2198-2208

* Originals not seen

Annexures

ANNEXURE I

Composition of MS basal medium (Murashige and Skoog, 1962)

Components	Quantity (mgl^{-1})
a) Major nutrients (Stock solution I)	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
b) Minor nutrients (Stock solution II)	
H_2BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Stock solution III	
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
d) Organic constituents (Stock solution IV)	
Myoinositol	100
Pyridoxine HCl	0.5
Glycine	2.0
Thiamine	0.1
Nicotinic acid	0.5
e) Sucrose	30 gl^{-1}
f) Agar	8 gl^{-1}

ANNEXURE II

Composition of bacterial culture media

Constituent	NA (gl^{-1})	YEM (gl^{-1})	YEB (gl^{-1})	LBA (gl^{-1})
Beef extract	1	-	5.0	-
K_2HPO_4	-	0.5	-	-
Yeast extract	2.0	1.0	1.0	5.0
MgSO4.7H2O	-	0.2	0.5	-
Peptone/Tryptone	5.0	-	5.0	10.0
Mannitol	-	10.0	-	-
NaCl	5.0	0.1	-	10.0
Sucrose	-	-	5.0	-
Agar	15.0	20	20	20

Adjust pH to 7.0

ANNEXURE III

1. Reagents used for Gram staining

1. Crystal violet/Gram stain

Solution A

Crystal violet (90% dye content)	- 2 g
Ethyl alcohol (95%)	- 20 ml

Solution B

Ammonium oxalate	- 0.8 g
Distilled water	- 80 ml

Mix solution A & B

2. Gram's iodine

Iodine	- 1 g
Potassium iodide	- 2 g
Distilled water	- 300 ml

3. Ethyl alcohol

Ethyl alcohol (100%)	- 95 ml
Distilled water	- 5 ml

4. Safranine

Safranine O	- 0.25 ml
Ethyl alcohol	- 10 ml
Distilled water	- 100 ml

ANNEXURE IV

Composition of reagents used for DNA isolation

1. **Lyzozyme stock**

   Lyzozyme - 1 mg

   Distilled water - 1 ml

   Stock was prepared by dissolving 1 mg lyzozyme in 1 ml water and was stored under refrigerated condition.

2. **0.5 M EDTA (pH-8.0)**

   EDTA - 18.612g

   Distilled water - 100 ml

3. **10 per cent SDS**

   SDS - 10g

   Distilled water - 100 ml

4. **3M Sodium acetate (pH-8.0)**

   Sodium acetate - 24.61g

   Distilled water - 100 ml

ANNEXURE V

Composition of Buffers and Dyes used for gel electrophoresis

1. **TAE Buffer 50X**

242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA (pH 8.0)

2. **Ethidium bromide**

The dye was prepared as a stock solution of 10 mg/l in water and was stored at room temperature in a dark bottle.

3. **Loading Dye (6X)**

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

ANNEXURE VI

Chemical composition of reagents used for Cloning and Transformation

1. **Plasmid isolation solutions**

a) Solution I (Resuspension buffer)

50 mM glucose
25 mM Tris HCl (pH-8.0)
10 mM EDTA 55(pH-8.0)

b) Solution II (Lysis buffer)

2N NaOH
1 per cent SDS

c) Solution III (Neutralization buffer)

5M Potassium acetate (60 ml)
Glacial acetic acid (11.5 ml)
Distilled water (28.5 ml)

ANNEXURE VII

1. **Formalin: Acetic acid: Alcohol (FAA) composition**

Formalin (40%)	- 5 ml
Glacial acetic acid	- 5 ml
Ethanol (95%)	- 90 ml

ANNEXURE VIII

1. **Trypan blue composition**

Trypan blue	- 50 ml
Lactophenol	- 100 ml

2. **Lactophenol composition**

Lactic acid	- 10 ml
Phenol	- 10 ml
Glycerol	- 20 ml
Water	- 60 ml

ANNEXURE IX

Details of Sequence Data obtained from sequencing agency

KAU-EC1

ACGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATCTGCCAGT
TTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACC
GCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTA
CCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCG
CCAAGGTTATTAACCTTAACGCCTTCCTCCCCGCTGAAAGTGCTTTACAACCCGA
AGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAAT
ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGG
CTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCATTACCCCA
CCTACTAGCTAATCCCATCTGGGCACATCTGATGGCATGAGGCCCGAAGGTCCCC
CACTTTGGTCTTGCGACATTATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCC
CTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCGCTCGTCACCCGAG
AGCAAGCTCTCTGTGCTACCGCTCGACTTGCATGTGTTA

KAU-CC1

CGATTGGGCCGACGTCGCATGCTCCCGGCCTCATGGCCGCGGGATTGGGCGGAG
TGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGC
GATTCCGACTCCATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTT
TCTGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCTCTGTCCCTACCATTGTAGTA
CGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAACT
AAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAG
CTGACGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGCACCAATCCATCTC
TGGAAAGTTCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATT
AAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGT
CTTGCGACCGTACTCCCAGCGGCGAACTTAACGCGTTAGCTTCGATACTGCGT

GCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGCGTGACTACAAGGTATC
TAATCTGTTTGCTCCCCACGCTTTCTGCCTCATGTCATGTTGGTCAGGTACTGCCT
CCCCATGGAGGTTCCCCTGATTCTACCATTCCTGCTAACAAGAATTCCCTACCCTT
AACCACCCTAGTGTCAGATACACTGCATTCGGGGTGGGACAAGGTTTCCAGAATT
AAACACCCCTACCCCCTTAACCGAATTCACCTGGCCTTTTAACGGGGCGCGCAAA
TAGCGGCTATCTGGGAGG

KAU-CC2c

GCGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTCAGGCCTAACA
CATGCAAGTCGGACGGTAGCACAGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGACGG
GTGAGTAATGTCTGGGGATCTGCCCGATAGAGGGGGATAACCACTGGAAACGGTGGCTA
ATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTCACTATCGGATG
AACCCAGATGGGATTAGCTAGTAGGCGGGGTAATGGCCCACCTAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
ATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAA
TAACCTTGCCGATTGACGTTACCCGCAAAGAAGCACCGGCTAACTCCGTGCCACAGCCGC
GGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGGAAACGCACCCAGGCGG
TCTG

MANAGEMENT OF RECALCITRANCY IN *IN VITRO* CULTURES OF CASHEW (*Anacardium occidentale* L.)

By

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THESIS

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ABSTRACT

The present investigation on "Management of recalcitrancy in *in vitro* cultures of cashew (*Anacardium occidentale* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2006-2008. An attempt was made to assess and manage recalcitrance of *in vitro* cultures of cashew with respect to phenolic exudation, endophytic microbial contamination, to increase root induction with *Agrobacterium rhizogenes* and to improve transplantation success with treatment with AMF-PGPR consortium.

The present study confirms earlier reports on the possible survival of bacteria in endophytic or covert form in plant tissue cultures and demonstrates the essentiality of a sequential screening of cultures involving visual examination, indexing of medium followed by tissue-indexing to detect such bacteria. In the absence of indexing, bacteria harboured in cultures would escape detection as they are not suspected as contaminated. One endophytic bacterium named as KAU-EC1 and two covert bacteria named as KAU-CC1 and KAU-CC2 were isolated from *in vitro* cultures. Variability among these three bacteria was studied by cultural and morphological tests.

Polymerase chain reaction was performed for identification of the bacteria through amplification of 16S rDNA gene. 16S rRNA molecules contain both highly conserved regions and variable regions. The highly conserved regions provide priming sites suitable for polymerase chain reaction and sequencing applications. 16S rDNA gene was amplified using two universal bacterial primers: $16S_{43\text{-}63}$ and $16S_{1404\text{-}1387}$. The PCR product when checked on agarose gel indicated the presence of a band of 1.3 kb size.

16S rDNA gene from KAU-EC1, KAU-CC1 and KAU-CC2 were cloned in pGEMT vector, sequenced and analysed after vector and adapter editing. *In silico* analysis using bioinformatics tools revealed that the sequence of

KAU-EC1 showed 99 per cent homology with *Klebsiella pneumoniae* strain SA-D6-7 16S ribosomal RNA gene, the cloned sequences of KAU-CC1 showed 98 per cent homology with *Pantoea agglomerans* strain XW123 16S ribosomal RNA gene and KAU-CC2 showed 93 per cent homology with *Stenotrophomonas maltophila* strain H2S8 16S ribosomal RNA gene. Three fungal species were detected 2, 4 and 6 weeks after culturing of nodal segments derived from field plants. The fungal species were identified as *Fusarium oxysporum*, *Fusarium moniliformae* and *Botryodiplodia theobromae*.

To standardize effective measures to adsorb phenols, those leached out into sterile water and liquid media (1/2 MS + 0.4 gl^{-1} glutamic acid) were estimated at different time periods. Friedman two way analysis of variance of ranks was carried out to detect the differential leaching of phenols into liquid media and sterile water. The highest mean rank score of phenols leached into liquid media was obtained for 0.2 per cent PVP followed by 0.5 per cent activated charcoal, followed by 100 mg ascorbic acid and control. Among the four treatments, 0.2 per cent PVP was found to be effective to adsorb phenols.

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An efficient method for *in vitro* plant regeneration was developed in *Anacardium occidentale*. In order to standardize a regeneration protocol, MS medium supplemented with varying concentrations of auxins and cytokinins were tried on different explants. Explants such as cotyledonary nodes, nodal segments and shoot tips from *in vitro* germinated seedlings and nodal segments from field grown plants were used for the study. Eighty per cent of the seeds germinated within 15 days in MS medium supplemented with 3.5 mg l^{-1} kinetin + 1 mg l^{-1} NAA, 3 per cent sucrose and 0.05 per cent activated charcoal.

Survival and bud break per cent of nodal segments derived from field plants after 5-6 weeks of culture was maximum during summer months especially April and lesser during June-July. Surface sterilization with 0.1 per cent $HgCl_2$ for 2 minutes was found to be optimum for culture establishment.

Maximum bud sprouting was observed in half MS medium. The establishment of nodal segments was more on MS medium supplemented with 400 mg l^{-1} glutamine and 0.2 per cent PVP and maximum multiplication was obtained on MS with 0.45 µM TDZ, 400 mg l^{-1} glutamine and 0.2 per cent PVP. Elongated shoots obtained from nodal segments of field grown plants failed to produce roots in the different media compositions tested.

Explants such as cotyledonary nodes, nodal segments and shoot tips derived from *in vitro* seedlings were also used. The multiplication of shoot buds derived from nodal segments and cotyledonary nodes were more on MS medium supplemented with 1.5 mg l^{-1} BA, 1 mg l^{-1} IAA and 0.05 per cent activated charcoal. Considering the formation and elongation of shoot buds together, maximum multiplication was obtained from MS medium supplemented with 2.5 mg l^{-1} BA, 1 mg l^{-1} IAA and 0.05 per cent activated charcoal. Shoot buds elongated well in MS medium supplemented with 2 mg l^{-1} BA, 400 mg l^{-1} glutamic acid and 0.05 per cent activated charcoal. The elongated shoot buds were successfully rooted in half MS with 1 mg l^{-1} IBA and 1 mg l^{-1} IAA by pulse treatment with 24 mg l^{-1} IBA for 24 hrs. Eighty per cent rooting was observed in this media.

Two strains of *Agrobacterium rhizogenes* (MTCC 2364 and MTCC 532) were evaluated to increase rooting of elongated shoots, but only thirty per cent rooting was observed with strain MTCC 2364.

The tissue cultured plantlets were inoculated with *Glomus fasciculatum* and *Bacillus subtilis* and their consortium during planting out. Maximum survival per cent (70.72%) was observed in the treatment involving *Glomus fasciculatum* and *Bacillus subtilis* followed by *Bacillus subtilis* with 68.35 per cent and then by *Glomus fasciculatum* with 66.68 per cent survival against 50.22 per cent recorded for control. Combined inoculation of *Glomus fasciculatum* and *Bacillus subtilis* resulted in maximum height and root growth of

the tissue cultured plantlets. Seventy per cent colonization was observed after 90 days of transplanting in the present study.

The 550 bp rDNA amplification product characteristic of AM fungi was consistently amplified from roots of tissue cultured plantlets colonized by *Glomus fasciculatum*. The plantlets were successfully hardened and transferred to large pots in the green house. The results of the study could be effectively utilized to manage recalcitrancy in cashew with respect to phenolic exudation, microbial contamination, low rooting and hardening success etc.