Agrobacterium rhizogenes mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L.

By

K. THIAGARAJAN

(2006.11.111)

THESIS

Submitted in partial fulfillment of the requirement for

the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

VELLANIKKARA THRISSUR-680 656

KERALA, INDIA

2010

DECLARATION

I hereby declare that the thesis entitled **"*Agrobacterium rhizogenes* mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title, of any other university or society.

Vellanikkara, K. Thiagarajan

(2006-11-111)

CERTIFICATE

Certified that the thesis entitled **"*Agrobacterium rhizogenes* mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L"** is a record of research work done independently by Mr. K. Thiagarajan under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellanikkara,

Dr. K. Surendra Gopal
(Major Advisor, Advisory Committee)
Associate Professor
Department of Plant Pathology
College of Horticulture
Vellanikkara

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. K. Thiagarajan** a candidate for the degree of **Master of Science in Agriculture**, with major field in **Plant Biotechnology**, agree that the thesis entitled **"*Agrobacterium rhizogenes* mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L"** may be submitted by **Mr. K. Thiagarajan** in partial fulfillment of the requirement for the degree.

Dr. K. Surendra Gopal
(Major Advisor, Advisory Committee)
Associate Professor
Department of Agricultural Microbiology
College of Horticulture
Vellanikkara

Dr. P. A. Nazeem
(Member, Advisory committee)
Professor and Head
Centre for Plant Biotechnology and
Molecular Biology (CPBMB)
College of Horticulture
Vellanikkara.

Dr. R. Keshavachandran
(Member, Advisory committee)
Professor
Centre for Plant Biotechnology and
Molecular Biology (CPBMB)
College of Horticulture
Vellanikkara.

Dr. D. Girija
(Member, Advisory committee)
Professor
Department of Agricultural Microbiology
College of Horticulture
Vellanikkara.

External Examiner

Acknowledgement

Words cannot express my deep sense of gratitude and indebtedness to **Dr. K. Surendra Gopal,** Associate Professor (Microbiology), Department of Plant Pathology and chairperson of my Advisory Committee. I wish to place my heartfelt thanks to him for his inspiring guidance, untiring help, patience, encouragement, constructive criticism and valuable suggestions during the period of the investigation and preparation of the thesis. My sincere and heartfelt gratitude ever remains with him.

I express my sincere gratitude to **Dr. P.A. Nazeem,** Professor and Head, CPBMB and member of my advisory committee for her valuable suggestions and guidance rendered me for the completion of the research programme and preparation of the thesis.

I am ever grateful to **Dr.R. Keshavachandran,** Professor, CPBMB and member of my advisory committee for his invaluable help, guidance and critical assessment throughout the period of the work. I thank his for all the help and cooperation he has extended to me.

I am deeply obliged to **Dr.D. Girija,** Professor, CPBMB and member of my advisory committee for her unfailing support and enthusiasm, relevant suggestions and whole hearted cooperation throughout the period of investigation.

I wish to express my sincere thanks to **Dr. Sally K. Mathew,** Professor and Head, Department of Plant Pathology for providing facilities to carry out the research work.

I also avail this opportunity to pay my sincere obligations and heartfelt thanks to **Dr.A. Augustin sir, Dr. P. C. Rajendran sir, Dr. P. A. Valsala madam and Dr. R. Sujatha madam** of CPBMB for their encouragement and kind help offered at different stages of the study.

I wish to express my sincere thanks to **Dr. A. N. Balakrishna**, Professor, UAS, Banglaore for providing *Glomus intraradices* mother culture to carry out the research work.

I wish to express my thanks to all the non-teaching staff members and labourers of CPBMB for their whole hearted cooperation and timely assistance.

Special thanks go to **Dr. S. Karthikeyan**, Associate Professor (Microbiology), Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai for providing valuable references whenever needed.

My special thanks to **Department of Biotechnology, New Delhi** for providing **JNU Fellowship** for successful completion of my M. Sc. Degree programme.

I am in dearth of words to thank my friends, **Manikandan, Nataraj, Sathishraj, Dr. Rathish, Koty sir, Sivaji, Dhinesh, Madhusudhan, Jaba, Devi Prasad, Labade Dinesh, Rahul, Kiran, Sampath, and Thamarai** for their encouragement and unflinching support.

With gratitude and affection, I recall the boundless affection, constant encouragement, warm blessings and motivation from my parents, brother without which this endeavor would never have become a reality.

(K. THIAGARAJAN*)*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

Introduction

1. INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) is an endomycorrhiza used as a biofertilizer and biocontrol agent in agriculture, horticulture and forestry. It helps in mobilisation and uptake of mineral nutrients, especially the less mobile forms of P, N, Cu, Zn etc. It also helps to reduce plant root diseases, especially root wilt and rot diseases and has a positive role in imparting stress tolerance (biotic and abiotic) in plants. However, the efficiency of AMF depends on several factors particularly the quality of inoculum. The mycorrhizal inoculum must be pure, viable and less bulky.

At present, pot culture, hydroponic culture, aeroponic and *in vitro* culture systems are being used but with little emphasis on large scale inoculant production. So far, no large scale multiplication method had been developed for AMF inoculant production, because it is obligate biotrophic nature it is difficult to meet the strict symbiotic conditions and aseptic environment. Pot culture is the most common method of AMF inoculant production but it is difficult to avoid bio-contamination during mass multiplication and also large scale production of inoculants. Modification of the physical and chemical nature of culture substrates can regulate the host-AMF symbiotic relationship. Methods of hydroponic and aeroponic culture are difficult to adopt as a method for routine AMF production due to its slow growth. Other methods such as *in vitro* aseptic culture and root organ culture need complex technology with little or no commercial inoculant output which makes it unviable.

AMF mass multiplication needs a host root which multiplies at a fast rate and produce high root biomass. In recent years, emphasis has been given to mass multiply AMF in hairy roots induced by *Agrobacterium rhizogenes* under *in vitro* conditions. One of the advantages with *A. rhizogenes* mediated hairy roots is its fast growth and high root biomass. Moreover, the AMF multiplied on hairy roots can be maintained as viable pure culture for a long period of time. Hence, a study was conducted with an objective to mass multiply *Glomus intraradices* in the *A. rhizogenes* mediated hairy roots of *Artemisia annua*.

Review of Literature

2. REVIEW OF LITERATURE

The literature on AMF importance, mass multiplication, hairy root induction and establishment of micropropagated plants using AMF are reviewed in this chapter.

2. 1 Effect of AMF on plant growth

Arbuscular mycorrhizal fungi (AMF) are soil fungi which form symbiotic association with plants and colonise roots of many 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) which imparts a variety of benefits to their hosts, which includes increased growth and yield due to enhanced nutrient acquisition (Diedrichs and Moawad, 1993), improved 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). Rathore *et al.* (2007) reported that dual inoculation of *G. fasciculatum* and *G. mosseae* resulted in the maximum plant height (18.65 cm), collar diameter (3.01 mm), total dry weight (1.31g) and quality index (0.173), in *Santalum album* seedlings followed by plant treatment with mixture of VAM spores.

Patil (2007) reported, per cent root colonisation by AM fungi in brinjal plant after 90 days of inoculation with *G. fasciculatum* and *G. mosseae*. Plants inoculated with *Acaulospora laevis* + *G. fasciculatum* + *G. mosseae* showed highest increase in fresh weight, dry weight, plant height and leaf area after 90 days of inoculation. *G. mosseae*, treated plants alone recorded highest number of flowers and stem diameter was maximum in *G. fasciculatum* treated plants after 90 days of inoculation.

2. 1. 1 Enhanced nutrient uptake

The improved plant growth due to mycorrhizal colonisation is mainly attributed to increased nutrient uptake especially phosphrous (Mosse, 1973; Harley and Smith, 1983; Jaizme – Vega and Azcon, 1991; Vidal *et al.,* 1992; Joseph, 1997;

Sivaprasad *et al.,* 1999a; Khaliq *et al.,* 2000; Estrada-Luna and Davies, 2003; Sivaprasad and Sulochana, 2004). It was due to fungal hyphae spread in the soil which increases the absorbing surface available for soil nutrient uptake for effective exploration of higher volume of soil for nutrients (Rhodes, 1980; Bolan, 1987). The role of AMF in improving the uptake of other nutrients such as N (Vidal *et al.,* 1992; LiMin *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 – Chavez *et al.,* 2002), Ni (Guo *et al.,* 1996; Jamal *et al.,* 2002) and U (Rufykiri *et al.,* 2002) are well documented. The nutrient elements recorded highest increase in N by *Acaulospora laevis*, P, K and Cu by *Acaulospora laevis* + *G. fasciculatum* + *G. mosseae*, S and Zn by *G. fasciculatum* and Fe in *G. mosseae* (Patil, 2007). Boby *et al.* (2007) reported, significantly higher microbial biomass and enhanced enzyme (dehydrogenase, urease, acid and alkaline phosphatase) activity in plants treated with *G. mosseae* when compared with uninoculated plants. The effect was much more pronounced when *G. mosseae* was inoculated with *Rhodotorula mucilaginosa* which resulted in highest microbial biomass and soil enzyme activity in the root zone soil.

2. 1. 2 Enhanced water uptake

AMF plays an important role in economising the water use of plants (Safir *et al.,* 1972; Al – Kraki, 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). AMF colonisation allowed plants to maintain their water content, water potential and leaf transpiration at high levels (Sanchez – Blanco *et al.,* 2004; Yano – Melo *et al.,* 1999). Jeffries *et al.* (2003) showed that AMF are the most important microbial symbioses, which influence plant community development, water relations and above ground productivity.

2. 1. 3 Drought tolerance

AMF associated plants enhanced tolerance of plants to toxicity and drought (Atkinson and Davidson, 1972; Guttay, 1976; Ruiz – Lozano *et al.,* 1999; Davies *et al.,* 2002). Mycorrhiza induced drought tolerance can be related to factors influenced by AMF colonisation such as improved leaf water and turgor potentials (Al – Karakki, 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 colonisation improved water use efficiency and plant yield in watermelon (Kaya *et al.,* 2003).

2. 1. 4 Well developed root system

Host plant roots show increased lateral root number and root length due to endomycorrhizal infection (Schellenbaum *et al*., 1991). AMF inoculation stimulates rooting and enhances root production of plants, which resulted 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 colonised micropropagated sugarcane plants showed excessive root growth and root dry weight, which results in better survival of these plants (Gosal *et al.,* 2001).

2. 1. 5 Salt and heavy metal tolerance

Arbuscular mycorrhizal fungi can accelerate the revegetation of severely degraded lands such as coal mines of waste sites containing high levels of heavy metals (Marx, 1975). 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 efficient fungus for bioremediation of heavy metal contaminated soils. AMF association were reported to be present on the roots of plant growing on heavy metal contaminated soils and hence play an important role in metal tolerance. Accumulation and isolation of these indigenous and stress-adapted AMF can be a potential tool for inoculation of plants for successful restoration of degraded ecosystems (Gaur and Adholeya, 2004).

2. 1. 6 Improved soil structures

Arbuscular mycorrhizal fungal hyphae plays an important role in erosion control by binding soil particles thereby maintaining soil stability (Miller and Jastrow, 1990). AMF enhances revegetation of degraded soils like mine soils (Pfleger *et al.,* 1994) by soil stabilisation (Bethlefalvay and Newton, 1991) and plant growth promotion due to AM symbiosis (Comprubi *et al.,* 1990). Rao and Tak (2002) observed that soil inoculation with *G. mosseae* significantly enhanced plant growth and biomass production in limestone mine spoils. AM fungi improved soil texture by binding soil particles into stable aggregates that resist wind and water erosion (Rillig and Steinberg, 2002; Steinberg and Rillig, 2003).

2. 2 Problem and approaches associated with 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 host roots. An effective AM fungal inoculum should promote significant growth, free of pathogens and should be economically produced with sufficient shelf - life. The quality of AMF inoculum produced should be pure and viable. The most common techniques used for AMF inoculum production are soil based inoculum, soil-less substrate based inoculum, hydroponic and aeroponic culture (Mosse and Thompson, 1984; Jarsfer and Sylvia, 1994). In most of these techniques, the inoculum gets mixed with pathogenic and saprophytic microorganisms. 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. Recently, attempts were made to make use of transformed root culture technique using *Agrobacterium rhizogenes* for the AMF inoculum production under aseptic conditions and met with considerable success (Mugnier and Mosse, 1987; Becard and Fortin, 1988; Chabot *et al.,* 1992; Khaliq and Bagyaraj, 2000; Potty, 2001; Tiwari and Adholeya, 2002).

2. 3 Hairy root disease

A large number of fine hairy roots and root hairs originate directly from the explant in response to *Agrobacterium* infection. Ricker *et al.* (1930) described and named the hairy root causing organism as *Phytomonas rhizogenes* which was later renamed as *Agrobacterium rhizogenes*. The transformation of higher plants using *A. rhizogenes* was made by Ackermann (1977).

2. 3. 1 Mechanism of *Agrobacterium* and plant interaction

The soil born gram negative bacteria *Agrobacterium* infects the crown region of the plant. The process of infection starts with attachment of bacterium to the plant. Wounding, induce the expression of *vir* genes plasmid (Binns and Thomasshow, 1988). The interaction between the bacteria and plant is a multistep process involving recombination, replication and repair activities most likely mediated by host cell enzymes (Zambryski, 1988). Integration and expression of T – DNA genes in host plant leads to the development of hairy roots (Vanhala *et al.,* 1995; White and Nester, 1980; Tepfer, 1984). Bacterium transfers its transfer DNA (T-DNA) which is a portion of large plasmid called root inducing plasmid (Ri - plasmid). Plasmid

contains *vir* – genes that are responsible for virulence of bacteria together with chromosomal genes and cause transfer of T-DNA. The border sequences (25 bp) of Ri – plasmid is determining the mobility of T-DNA (Sevon and Oksman – Caldentey, 2002). Steps involved in transfer of genetic material from *Agrobacterium* to plant cell are (Bapat and Ganapathy, 2005):

- Wounding of plant cell and secretion of the phenolic compounds
- Detection of the wound signal by the bacterium
- Activation of the bacterial gene machinery
- Induction of *vir* gene expression
- Generation of T-DNA
- Formation of T-DNA strand - protein complex
- Movement of T-DNA complex through bacterial cell membranes
- Targeting of the T-DNA complex into and within the plant cell
- Targeting of the T-complex into cell nucleus and its stabilisation
- Integration of the T-DNA strand into plant cell DNA at random
- Expression of the bacterial DNA in the plant cell

2. 3. 2 *Agrobacterium rhizogenes* mediated genetic transformation

A large number of plant species from a number of families have been successfully transformed by *A. rhizogenes*. Ooms *et al*. (1985) reported that Ri plasmids could be used as vectors to introduce any gene via Ri T – DNA into potato. The use of *Agrobacterium* as a vector is based on its unique capacity to transfer a piece of its own DNA (T - DNA) into nuclear genome of plant cells. *A. rhizogenes* strains are more virulent than *A. tumefaciens* strains. It has been shown in several species that shoots can easily be regenerated from transformed hairy roots (Noda *et al.,* 1987; Ottaviani *et al.,* 1990). *A. rhizogenes* can be used to insert non T- DNA genes into plants (Tepfer, 1990). A set of morphological markers is encoded by Ri T_L – DNA in regenerated plants (Ackermann, 1977; Durang – Tardif *et al.,* 1985; Sinkar

et al., 1988). These include wrinkled leaves, short internodes, altered flower morphology etc. Some of these are observed in particular species in certain clones within species and some only in certain individuals (Tepfer, 1984; Durang – Tardif *et al.,* 1985). These markers make up transformed phenotype and segregate with Ri T – DNA after meiosis (Tepfer, 1984). The transformed phenotype provides a simple and accurate way of identifying transformed materials. The *A. rhizogenes* induced hairy root transformation in the genus *Phaseolus* triggers functional genomics programme focused on root physiology, root metabolism, and root–microbe interactions (Navarrete *et al.,* 2006).

2. 3. 3 Characteristics of hairy roots

2. 3. 3. 1 Rapid growth

Hairy roots have more number of apical zones with high degree of cell division **(**Bapat and Ganapathy, 2005**).** High level of lateral branching in hairy roots due to presence of many lateral root tips resulted in high growth rate (Sevon and Oksman – Caldentey, 2002; Xu *et al.,* 2004) without growth regulator in the media (Payne *et al*., 1992). There is difference in growth of hairy roots among different species and between different root clones of the same species (Sevon and Oksman – Caldentey, 2002).

2. 3. 3. 2 High stability

The attractiveness of hairy root cultures in bioprocessing can be attributed to genetic and biochemical stability (Wysokinska and Chmiel, 1997; Li *et al.,* 2000).

2. 3. 4 Culture medium for *A. rhizogenes* strains

Benjamin *et al.* (1993) reported the use of AB minimal media for culturing 15834 strains of *A. rhizogenes*. Mano *et al.* (1986) suggested nutrient broth as a good culturing medium for ATCC 15834, A_4, NCPB 1855 and NCPB 2659 strains. YEB

medium was found to be the best media for the growth of MAFF 03 –01724 (Sauerwein *et al.,* 1992; Jaziri *et al.,* 1994; Momocilovic *et al.,* 1997; Chen *et al.,* 1999; Shi and Kintzios, 2003; Yoshimatsu *et al.,* 2003; Xu *et al.,* 2004; Zdravkovic – Korac *et al.,* 2004). LB medium was used for culturing *A. rhizogenes* strains 15834 (Dobigny *et al.,* 1995; Lee *et al.,* 2004) AR – 12 (Arellano *et al.,* 1996) 2659 (Dobigny *et al.,* 1995). YMB medium (1% Mannitol, 0.05% K_2HPO_4, 0.01% NaCl, 0.04% Yeast extract, 0.02% $MgSO_4.7H_2O$) were used for culturing *A. rhizogenes*. ATCC 15834 strain (Hu and Alfermann, 1993; Vanhala *et al.,* 1995) LBA 9402 (Hu and Alfermann, 1993; Allan *et al.,* 2002; Chaudhuri *et al.,* 2005; Van-Wordragen *et al.,* 1992, Ooms *et al.,* 1985) A_4 **(**Hu and Alfermann, 1993; Chaudhuri *et al.,* 2005). *A. rhizogenes* strains A_4, R 1022, K 599 and SA 79 were grown in yeast extract peptone medium (Batra *et al.,* 2004). *Agrobacterium rhizogenes* such as ATCC 15834, A4, WC and WR were grown on YEB medium (Chandran and Potty, 2008). Rahimi *et al.* (2008) reported that the addition of 100µM acetosyringone to YMB growth medium enhanced the inducing ability of *A. rhizogenes*.

2. 3. 5 Culture conditions for *A. rhizogenes* strains

Temperature has influence on the growth of *A. rhizogenes* strains. The optimum temperature range is 25 ± 3°C. Jaziri *et al.* (1994) suggested 25°C for culturing strains like ATCC 15834 and MAFF 03-01724. Optimum temperature for growth of *A. rhizogenes* strains like ATCC 15834, A_4 and LBA 9402 were found to be 28°C (Shi and Kintzios, 2003; Momocilovic *et al.,* 1997; Celma *et al.,* 2001; Lee *et al.,* 2004). Xu *et al.* (2004) reported that A_4, R 1601 and ATCC 15834 were cultured at 27°C and the culture media was shaken at 150 rpm.

2. 3. 6 Explants used for hairy root induction

The susceptibility of plant species to *Agrobacterium* strain varies with explants. Xu *et al.* (2004) reported that root induction efficiency was influenced by the type of explants *A. rhizogenes* strains, the age and differentiation status of plant

tissue and plant type. The level of tissue differentiation also determines the ability to give rise to transformed hairy root after *A. rhizogenes* inoculation (Trypsteen *et al.,* 1991). Karmarkar (2001) used hypocotyls and shoot buds for transformation in *Holostemma ada-kodien* using *A. rhizogenes* strains A_4, ATCC 15834 and pCA_4. Leaf segments and shoot tips showed efficient transformation in *Withania somnifera* using *A. rhizogenes* (Varghese, 2006). Stem explants of tomato are found to be best suited for root induction and A. *rhizogenes* strains LBA – 9402, ATCC 15834 A_4 gave maximum callus induction and root initiation (Dayala, *et al.,* 2007). Hairy roots were induced from hypocotyl explants excised from seven - day - old aseptically grown seedlings of groundnut using *A. rhizogenes* 15834 (Karthikeyan, *et al.,* 2007)**.** Induction of hairy roots by four strains of *A. rhizogenes* - ATCC 15834, A_4, WC and WR were studied in five plants viz., *Ipomoea batatas*, *Solenostemon rotundifolius*, *Vigna vexillata*, *Pachyrrhizus erosus* and *Canavalia* species. Among the five plants, *P. erosus* was found resistant to all the four bacterial strains (Chandran and Potty, 2008). They reported hairy roots induction from the cotyledons, hypocotyls, stem cuttings and *in vitro* plants of *I. batatas* through the transformation of 15834 and A_4 strains of *A. rhizogenes*. A method for induction of hairy roots of four *Hyoscyamus* species (*H. arachnoideus* Pojark., *H. kurdicus* Bornm., *H. reticulates* L. and *H. squarrosus* Griff.) were established by infecting leaf discs with five strains of *Agrobacterium rhizogenes* (1724, 2659, 15834, A4 and LBA 9402) in MS and B5 media (Ackramian *et al.,* 2008). The successful induction and proliferation of hairy roots of *Valeriana sisymbriifolium* explants through the transfer of *rolA* gene by *A. rhizogenes* (ATCC 15834) was reported by Rahimi *et al.* (2008). Using different explants of *in vitro* seed grown *Scutellaria baicalensis* (var. Georgi) plantlets, hairy roots were induced following inoculation of *Agrobacterium rhizogenes* strains A4GUS, R1000 LBA 9402 and ATCC11325 (Tiwari *et al.,* 2008). Hypocotyls, callus and shoots of *Ruta graveolens* were inoculated with two strains of *Agrobacterium*

rhizogenes and hairy root cultures were established after inoculation of hypocotyls with wild *A. rhizogenes* strain LBA 9402 (Matylda Sidwa-Gorycka *et al.,* 2009).

2. 3. 7 Pre culturing of explants

Yoshikawa and Furuya (1987) used the co – culture method to obtain hairy roots. Cultured cells of ginseng were partially digested with cellulose and pectinase and incubated with *A. rhizogenes*. Momocilovic *et al.* (1997) reported that shoots of *Gentiana acualis* were elongated on basal media with 34.6 mg l^{-1} GA3 for 4 weeks prior to inoculation with *A. rhizogenes*. Shi and Kintzios (2003) reported pre - culturing of *Pueraria phaseolides* leaf. Leaves were cut in to pieces with or without petiole and were pre - cultured on MS solid media without growth regulator for 24 hours. Xu *et al*. (2004) reported that leaves (0.5 cm^3 blocks) and hypocotyls (1cm long) explants of *Isatis indigolica* were pre - cultured for 2 days in 12 hour light – dark period. Leaves from one - month - old *in vitro* grown plants of *Catharanthus roseus* were incubated on half strength Gamborg's medium for 24 hrs (Batra *et al.,* 2004).

2. 3. 8 Wounding of explants

Wounding is a pre - requisite for *Agrobacterium* infection. According to Hilderbrand (1934), *A. rhizogenes* has to enter a wound deep enough to reach the phloem region to induce hairy roots on apple trees. Moore *et al*. (1974) showed that most hairy roots emerged from the pericycle tissue of carrot. Stem of potato shoots were punctured with a Pasteur pipette and the wound sites were infected with *A. rhizogenes* (Ooms *et al.,* 1985). Leaves of *Salvia miltiorrhiza* were wounded with forceps (Hu and Alfermann, 1993). Only cells containing high levels of auxin and sucrose are able to act as root meristem initials and are ideal targets for *A. rhizogenes* infection (Nilsson and Olsson, 1997).

Pawar and Maheswari (2004) wounded leaf discs of *Withania somnifera* using a scalpel. Embryos (10 to 20 mm) at cotyledonary stage of *Aesculus hippocastanum* were wounded by puncturing with a hypodermic needle to a depth of 1 to 2 mm, which was found to be more efficient than mere scratching. Since, ray cells and phloem cells are positioned in the region with high amount of sucrose and considerable amount of IAA, they could be convenient targets for *A. rhizogenes* (Zdravkovic-Korac *et al.,* 2004). Leaf segment of *Holostemma ada-kodien* were prepared by cutting the leaf margins and ten pricks were made on the lower side of leaf on the leaf lamina and mid rib, 15 pricks were made at shoot buds and inter nodal segments (Karmarkar and Keshavachandran, 2005) to induce hairy roots.

2. 3. 9 Inoculation and co – culturing of explants with *A. rhizogenes*

Hawes *et al*. (1988) reported that motile strains of *Agrobacterium* exhibited virulence only in liquid medium but mutant strains (non motile) exhibited virulence when inoculated directly on wounds. Hu and Alfermann (1993) reported that leaf explants of *Salvia miltiorrhiza* were infected with small droplets of bacterial suspension. Elongated shoots of *Gentiana acaulis* were inoculated by puncturing internodes or central leaf nodes with a hypodermic needle dipped in bacterial suspension or bacterial suspension was smeared on cut surface of decapitated shoot (Momocilovic *et al.,* 1997) and kept for incubation for 48 h.

Azadirachta indica leaves were infected by scratching with a sterile needle dipped in dense *Agrobacterium* suspension and incubated in MS medium in dark at 25^{o}C (Allan *et al.,* 2002). Leaf explants of *Pueraria* were infected by dipping them into *Agrobacterium* suspension in MS medium for 20 min (Shi and Kintzios, 2003) and were co – cultured at 28^{o}C for 2 days. Cotyledon and hypocotyls explants of *Isatis indigotica* were immersed in bacterial suspension at exponential growth phase (OD_{660} ~ 0.7) for ten min and co - cultured in dark for two days on MS basal medium (Xu *et al.,* 2004). The wounded embryos of *Aesculus hippocastanum* were immersed

in bacterium suspensions for 5 min and they were transferred to MS medium with or without 50µ*M* acetosyringone and co – cultured for 72 h (Zdravkovia – Korac *et al.,* 2004).

The wounded explants *H. ada – kodien* were inoculated with 48 h old cultures of *A. rhizogenes* directly by applying a drop of bacterial suspension on each wound. The explants were then co – cultured for 24 hrs (Kamarkar and Keshavachandran, 2005). *Tylophora indica* explants were wounded at different sites with a hypodermic needle loaded with 10 ml of bacterial suspension. Excised leaf explants were wounded at node or internode and incubated for 72 hrs (Chaudhury *et al.,* 2005). Pre - incubated leaves of *Catharanthus roseus* were infected with bacterial culture (OD_{600} - 0.8 – 1.0) by multiple pricks with a syringe and the infected leaves were incubated in the dark (Batra *et al.,* 2004).

2. 3. 10. Co - culture and conditions for hairy root induction

The leaf explants of *Salvia miltiorrhiza* after infection with bacteria were cultured on MS medium with 1g l^{-1} casamino acid, 2 per cent sucrose and 0.8 per cent agar containing 500 mg l^{-1} cefotaxime. Culture was maintained at 25^{o}C in dark (Hu and Alfermann, 1993). Medium had a significant effect on root formation when compared to B_5 media. However, hairy root formation was faster on B_5 than on MS medium, particularly in case of LBA 9402. The low salt concentration in B_5 medium favoured all the bacteria to spread on the medium. The wounded leaves of *Hyoscyamus muticus* were transferred to basal medium containing 500 mg l^{-1} carbenecellin (Vanhala *et al.,* 1995).

The affected potato explants were sub cultured on MS basal medium with 500 mg l^{-1} cefotaxime (Dobigny *et al.,* 1995). The infected explants of *Gentiana acaulis* after 48 h were transferred to basal medium supplemented with 200 mg l^{-1} cefotaxime (Momocilovic *et al.,* 1997).

2. 3. 11 Efficiency of *A. rhizogenes* inducing hairy root

The plant species which were shown to be insusceptible to one *A. rhizogenes* strain have been successfully transformed with other strains (Jung and Tepfer, 1987). It has been reported that virulence of *Agrobacterium* strains varies among plant hosts (Hobbs *et al.,* 1989; Bush and Pueppke, 1991) and that transformation efficiency of host species vary among different bacterial strain (Godwin *et al.,* 1991; Hu and Alfermann, 1993). Significant differences were observed among transformation ability of differential strains of *Agrobacterium* (Vanhala *et al.,* 1995). Jung and Tepfer (1987) reported that *A. rhizogenes* strain 8196 is better adapted to root initiation in *Calystegia sepium* and A4 is better suited to *Atropa belladonna*. Sauerwein *et al.* (1992) reported that maximum yield of alkaloid *Hyoscyamus albus* transformed with A4 when compared to 15834 and MAFF 03.01724. Hu and Alfermann (1993) reported LBA 9402 strain caused 85 per cent rooting in leaf explants of *Salvia miltiorrhiza* when compared to A_4 (10 %) and ATCC 15834 (20 %).

Mannopine and cucumopine strains were unable to induce root formation in potato. So, NAA pre - treatment was done to stem fragments. The strain 15834 was poor in inducing root formation (Dobigny *et al.,* 1995). Xu *et al*. (2004) reported that the strain A4 was better when compared to R 1601 and ATCC 15834 in root induction efficiency from a cotyledon which was higher than hypocotyls. Lorence *et al.* (2004) tested the ability of two different strains of *A. rhizogenes*, ATCC 15834 and R 1000 on inducing hairy root in *Camptotheca acuaminata*. They found that the strain 15834 infected more than 40 to 45 per cent of explants, but in contrast, strain R 1000 infected only 20 to 24 per cent of exposed tissue. Chaudhuri *et al.* (2005) reported that *Tylophora indica* is more susceptible to strain A_4 than to strain LBA 9402. Among the five *A. rhizogenes* strains evaluated, PcA4, ATCC 15834 and A_4 induced hairy roots in *Holostemma ada – kodien* whereas 8196, 2659 did not induce hairy roots at all (Karmarkar and Keshavachandran, 2005). Of the three strains, A_4,

ATCC 15834 and MTCC 2364, ATCC 15834 and A4 were able to induce hairy roots in *Withania somnifera* (Varghese, 2006).

2. 3. 12 Establishment of hairy root cultures

In most cases, hairy roots emerge within 1 to 4 weeks. The transformed roots of potato were excised and grown on solidified MS media with 2 per cent sucrose and 200 $\mu g ml^{-1}$ cefotaxime or carbenecellin (Ooms *et al.,* 1985). Roots obtained from *Solanum* sp. transformed with A4 strain were excised and individual root tips were transformed to petridishes containing MS medium with 500 $\mu g\ cm^{-3}$ cefotaxime (Devey *et al.,* 1987). The hairy roots emerged from *Acmella oppositifolia* were transferred to basal solid media containing 100 to 200 $mg l^{-1}$ carbenecillin (Flores *et al*., 1993).

Segments of hairy roots of *Perezia cuernavacana* were excised and cultured on hormone free MS liquid with 30 $g\ l^{-1}$ cefotaxime at 80 rpm (Arellano *et al.,* 1996). The adventitious roots of *Gentiana acaulis* (1.5 cm long) were excised and about 5 root tips were transformed to petridish containing 30 ml of basal medium with antibiotic (Momocilovic *et al.,* 1997). *Azadirachta indica* has produced single hairy roots of 2 to 3 cm long from leaf explants when co - cultivated with *A. rhizogenes* strains LBA 9402 in MS liquid medium containing 10 $mg\ l^{-1}$ ampicillin. Later, ampicillin content was decreased by 50 per cent upon each weekly transfer of whole roots (Allan *et al.,* 2002). The transformed roots of *Cichorium intybus* were cultivated in a modified liquid MS containing half strength macronutrients and 3 per cent sucrose supplemented with 500 $mg\ l^{-1}$ cefotaxime. Cultures were kept at 25^{o}C in gyratory shaker with 110 rpm. The roots were sub - cultured every four weeks by inoculating 0.7 g fresh weight of roots in 250 ml Erlynmeyer flask containing 30 ml of nutrient medium (Malarz *et al.,* 2002).

Roots of *T. indica* that formed at the wounded sites of infected explants were excised and cultured in dark in basal media with 500 $mg\ l^{-1}$ ampicillin. Each excised

primary root was propagated as a separated clone and sub cultured at 4 week interval. Hairy roots turned white to yellowish white then to reddish brown in 6 to 8 weeks (Chaudhuri *et al.,* 2005). Hairy roots of *Aesculus hippocastanum* were isolated and placed in MS plant growth regulator free liquid medium supplemented with 500 mg l^{-1} cefotaxime to inhibit bacterial growth shaken at 85 rpm and transferred biweekly. The concentration of cefotaxime was gradually lowered (Zdravkovia – Korac *et al.,* 2004).

2. 3. 13 Effect of culture media and conditions on growth of hairy roots

Several physical and chemical factors have been found to influence growth and productivity of hairy root cultures. Toivonen *et al.* (1991) found that biomass yield of hairy roots was maximum in media containing high sucrose and ammonia. Factors such as carbon source and its concentration, ionic concentration of medium, light (Christen *et al.,* 1992) phytohormone (Rhodes *et al.,* 1994) temperature (Toivonen *et al.,* 1991) and inoculum (Mano *et al.,* 1986) are known to influence the growth of hairy roots.

Growth rate of hairy roots produced by A_4 strain cultured in light was enhanced by addition of low concentration of phytohormones whereas addition of phytohormones reduced the growth of hairy roots transformed by *A. rhizogenes* MAFF - 03 - 01724.

The hairy roots of different species behave differently in the same culture conditions. Individual hairy root clones can also have different optimum concentrations of sucrose or mineral ions (Oksman - Caldentey *et al.,* 1994). Gamborg's B_5 medium is the most widely used medium for the hairy roots of many species (Hilton and Wilson, 1995). Xu *et al.* (2004) found that the culture medium have a significant effect on *Isatis indigotica* hairy root growth. Among four liquid media (MS, 1/2MS, B_5 and White's) tested, MS and half MS were found to be significantly superior to the other two and on comparison between B_5 and White's

medium, B_5 medium was significantly better than white's medium on hairy root growth. Half MS media with 3 per cent sucrose was found to be superior for promoting hairy roots in *W. somnifera* (Varghese, 2006).

2. 3. 14. Growth characteristics and morphology of hairy roots

Hairy roots are capable of fast growth in hormone free medium. The precise mechanism involved in lateral root formation is still not clearly understood, but roots transformed by *A. rhizogenes* are characterised by the spontaneous formation of numerous lateral, an important factor contributing to their high biomass productivity (Tepfer, 1984). Significant differences were observed in the transformation ability of different strains of *Agrobacterium* in *Hyosyamus muticus*.

The roots were formed mainly on the midrib of leaf and they differed morphologically depending on the bacteria. The A_4 strain inoculated roots were white, thin and long. The LBA 9402 roots were thin and white turning green. The ATCC 15834 roots were long, green in colour and it was difficult to remove bacterial growth (Vanhala *et al.,* 1995). Many variations in the morphology of transformed hairy roots have been reported. The correlation studies on the integration of genes of T_L-DNA and T_R-DNA regions with morphology of hairy roots and regenerated plants have been carried out (Hanishten – Cate *et al.,* 1990; Limani *et al.,* 1998; Christey, 2001). Hairy roots show morphological variation depending upon the interaction, nature of plant cell genotype, strain of the bacterium and showed differences in root thickness, degree of branching and amount of hairy root production (Bapat and Ganapathy, 2005). The A4, ATCC15834 and PcA4 induced hairy roots of *H. ada-kodien* were whitish in colour and showed negative geotropic growth (Karmarkar and Keshavachandran, 2005). The roots induced by ATCC 15834 were relatively thick with high root hairs compared to that of A4 strain, which produced relatively thin roots with less root hair in *W. somnifera* (Varghese, 2006).

2. 4. Hairy root induction in *Artemisia annua*

2. 4. 1 *Agrobacterium rhizogenes* strains used for hairy root induction in *A. annua*

Various strains are used to induce hairy roots under *in vitro*. Transformation efficiency is known to differ with different bacterial strains. Hairy root cultures of *A. annua* were established using *A. rhizogenes* strains MAFF03-01724 or NCIB 8196 (Jaziri *et al.,* 1995) and strains LBA 9402, 8196 and A_4 (Mukherjee *et al.,* 1995). Strain R-1601 was used for infecting *A. annua* (Cai *et al.,* 1995) and also ATCC-15834 (Liu *et al.,* 1998; Chen *et al.,* 1999).

Primary roots were visible within 7-21 days depending on the *A. rhizogenes* strain. *A. rhizogenes* strains A4, ATCC15834, K599, LBA-9402, 9365 and 9340 were used for induction of hairy roots in *A. annua*. The best root formation response as transformation frequency was achieved with *A. rhizogenes* strains in the order LBA 9402, 9340, 9365, 15834 and A4. Bacterial cultures induced with acetosyringone and explants co-cultivated on MS basal medium with acetosyringone reduced the time of hairy root induction by a week (Giri *et al.,* 2001).

2. 4. 1. 1 Explants used for transformation in *Artemisia annua*

Leaves from 6 to 8 week old *in vitro* grown plants (Jaziri *et al.,* 1995) or three week old seedlings (Chen *et al.,* 1999; Wang and Tan, 2002) were used as explants as they normally do not develop roots spontaneously in control experiments. Leaf, stem and root explants from 12 to 18 week old plants and cotyledons and hypocotyls from 8 - day - old seedling were used for transformation using *A. rhizogenes* in *A. annua* (Verguawe *et al.,*1998). Leaf, petiole sections and shoot tips from *in vitro* grown *A. annua* shoot cultures were used as explants for transformation (Giri *et al.,* 2001). Leaf segment showed efficient transformation in *Artemisia annua* using *A. rhizogenes* (Shaneeja, 2007).

2. 4. 2 Transformation methods

Bacterial colony on solid medium or bacterial suspension can be used for transformation. Hairy root cultures were established by co-culture method using leaf discs of *Artemisia annua* (Jaziri *et al.,* 1995). Mukherjee *et al.* (1995) produced hairy root cultures by infecting leaves of 6 to 8 week old plants as described by Ooms *et al.* (1985). Leaves from 2 to 3 week old aseptic seedling were floated in 8 ml liquid MS containing 2 ml of a late log phase *A. rhizogenes* in 50 ml Erlynmayer flask. After 20 min. of infection, explants were blotted with sterile filter paper and sub cultured on MS solid medium and kept at 25^0C for 2 days.

The explants were transferred to MS medium with 500 mg l^{-1} cefotaxime (Chen *et al.,* 1999). Explants were incubated for 20 min in actively growing bacterial culture of different *A. rhizogenes* strain on nutrient broth with or without acetosyringone and they were co - cultivated for 48 h (Giri *et al.,* 2001). *Agrobacterium rhizogenes* was grown overnight at 20^0C in liquid YEB media and pelleted by centrifugation. This was resuspended in liquid YEB and diluted 10 fold with MS liquid media and explants were incubated for 20 min (Wang and Tan, 2002).

2. 4. 3. Establishment of *Artemisia annua* hairy root cultures

Mukherjee *et al.* (1995) reported that hairy roots appeared at the point of inoculation within 2 weeks and these were transferred to liquid MS medium without growth regulators containing 500 mg l^{-1} ampicillin. The cultures were maintained at 25 ± 1^0C at 70 rpm. Control roots were maintained in MS medium with 0.2 mg l^{-1} IBA. The transformed roots of *A. annua* were cultured in half MS medium and maintained at 25^0C in dark on rotary shaker (100 rpm) (Jaziri *et al.,* 1995). The fastest hairy root growth was observed in one fourth MS (Giri *et al.,* 2001). Hormone free MS medium with 3 per cent sucrose were used for culturing hairy roots (Wang and Tan, 2002). Gonzalez and Weathers (2003) used B_5 media with 3 per cent sucrose for sub culturing of hairy roots.

2. 4. 4 Morphology of hairy roots

Hairy root clones of *A. annua* that were induced by *A. rhizogenes* NCIB 8196 turned green (Jaziri *et al.,* 1995) and by ATCC 15834, LBA 9402, turned green when exposed to light (Giri *et al.,* 2001). The hairy roots of *A. annua* turned brown if not sub - cultured and there was no spontaneous shoot regeneration from hairy roots (Mukherjee *et al.,* 1995).

2. 5 Factors affecting hairy roots formation

Young materials greatly enhances the transformation efficiency of hairy root formation (Rech *et al.,* 1989; Trypsteen *et al.,* 1991). Elevated temperatures (>20 - 22^{o}C) significantly lowered the transformation frequencies (Stiller *et al.,* 1997; Boisson - Dernier *et al.,* 2001).

2. 6 Transformed root culture for AMF inoculum production

The pathogenic condition known as 'hairy root' is caused on the host plant by *Agrobacterium rhizogenes* (Ricker *et al.,* 1930) Conn. (Ark and Thompson, 1961). The bacterium infects the sensitive host plant and stimulates excessive proliferation of roots (Chilton *et al.,* 1982) by transferring root inducing Ri T - DNA to plant (Schenk, 1982). The pioneering work on *in vitro* culture was initiated in the early 1960's. The use of Ri T - DNA transformed roots of *Daucus carrota* L. as a host by *Agrobacterium rhizogenes* resulted in an increase in spore production of *Glomus mossae* (Mugnier and Mosse, 1987).

Becard and Fortin (1988) developed an *in vitro* system using Ri T – DNA transformed carrot roots and vesicular arbuscular mycorrhizal fungus, *Gigaspora margarita* to study the initial events of mycorrhiza formation. Mugnier (1987) could obtain typical infections of *Polymyza betae* and *Plasmodiaphora brassicae* in root organ culture of their host; *Beta vulgaris* or *brassicae*, containing the root inducing transferred DNA of *A. rhizogenes*. Becard and Piche (1990) studied the physiological

factors determining AMF formation in non mycorrhizal host (Sugar beet) and mycorrhizal host (Carrot) Ri T – DNA transformed roots and found that sugar beet lack factors that promote mycorrhizal infection.

In addition to *G. margarita* and *G. mosseae*, other species of AMF that have been cultured in transformed roots are *G. intraradices* (Chabot *et al.,* 1992), *G. gigantia* (Douds and Becard, 1993). Leu *et al.* (1994) obtained *in vitro* sporulation of AMF, *G. gigantea* and *G. mosseae* by dual culture with carrot Ri T – DNA transformed root and tomato root culture. Bi Yin Li *et al.* (1999) studied the morphological characters of AM symbiosis between AMF and transformed Ri T – DNA carrot roots. Karandashov *et al.* (1999) investigated the pre requisites for *in vitro* culture of AMF together with plant hairy roots. Pawloska *et al.* (1999) reported the establishment of monoxenic cultures of *G. etunicatum* in association with excised Ri T – DNA transformed carrot roots. Khaliq and Bagyaraj (2000) also succeeded in making use of transformed root culture technique using *A. rhizogenes* for AMF inoculum production under aseptic conditions. *Medicago truncatula* hairy root explants can be propagated *in vitro* and demonstrated that these clonal lines can be colonised by endomycorrhizal fungi such as *Glomus intraradices* with the formation of arbuscules within cortical cells (Dernier *et al.,* 2001). Under *in vitro* conditions, low P concentrations and addition of N to the media reduced spore germination of *G. etunicatum*, stimulated root length, colonized root length and increased total root length. Under low N concentration, addition of P stimulated spore germination, root length, colonized root length and increased total root length (Bresson, 2001). Dalpe and Declerck (2002) for the first time successfully grew a strain of *Acaulopora rehmii* in Ri T - DNA transformed carrot roots under *in vitro* and allowing the *in situ* observation of *Acaulospora* spore development and extra radical thin walled hyphal swellings.

The method by which root organ cultures (transformed and non transformed) have been obtained, together with the choice of host species, inoculation techniques

and culture media and the considerable impact of *in vitro* root organ cultures on AMF morphology, taxonomy and physiology were described (Fortin *et al.,* 2002). Tiwari and Adholeya (2002) reported successful co – culture of two genera of AMF, *Glomus intraradices* and *Gigaspora margarita* under *in vitro* condition using Ri T - DNA transformed carrot root. Various stages in the production of hairy roots in a lesser known tuber crop like *Vigna vexillata* using *Agrobacterium rhizogenes* ATCC 15834 strain for culturing mycorrhizal fungi was reported by Potty and Pratapchandran (2004).

2. 7 Molecular detection of AMF

DNA amplification by PCR has been proposed as a technique for AM fungi identification (Simon *et al.,* 1993; Wyss and Bonfante, 1993). Highly conserved regions of the gene enable it to be sequenced rapidly either directly from rRNA or from DNA amplified by polymerase chain reaction with universal primers (Hamby *et al.,* 1988; Edwards *et al.,* 1989; Sogin *et al.,* 1989 and Bousquet *et al.,* 1990). A portion of the small – subunit 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 application for AM fungi have been carried out with spores or purified DNA from colonised leek roots (Simon *et al.,* 1992b). PCR was used with the primer pair VANS1-NS21 to detect the arbuscular mycorrhizal fungus *Glomus intraradices* on roots of lettuce, zinnia, leek, pepper and endive plants. (Bonito *et al.,* 1995). The AMF detection was accomplished without extraction of DNA from colonised root by simply boiling root tissue in a buffer (Henson *et al.,* 1993 and Bonito *et al.,* 1995). Bonito *et al.,* (1995) reported that addition of Chelex resin to the Tris - HCL buffer (when the colonised roots were boiled) resulted in increased amplification of the 550 bp AM rDNA product from reaction mixtures. The *Glomus intraradices* specific primer amplified as low as 1 pico gram of its target DNA, which was allowed them to detect a single spore of

fungus in host using real – time polymerase chain reaction (RT - PCR) (Alkan *et al.,* 2004).

2. 8 Effect of AMF on micropropagated plants

2. 8. 1 Physiological feature of tissue culture plants

The tissue culture plantlets when planted out undergo desiccation and drying due to poor development of cuticle and epicuticular on the newly emerging leaves as a result of high humidity in the culture vessels (Lesham, 1983). The palisade cells of leaf surface are poorly developed and have pronounced mesophyll air space (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 organisation 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). Lack of proper root hair development in tissue culture plants in general directly affects the absorption of nutrients and water form the soil (Reuther, 1986).

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). These major physiological defects of tissue culture plantlets affect uptake of water and nutrients from soil and transportation to the shoot system and the plantlets become more vulnerable to desiccation and wilting and also to pathogenic infection (Sivaprasad and Sulochana, 2004).

2. 8. 2 Integration of AMF with tissue culture plants

Studies have proved that successful hardening and *ex vitro* establishment of micropropagated plantlets could be achieved by inoculation with AMF at the time of

planting out (Lovato *et al.,* 1994). 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 *G. mosseae* (Fogher *et al.,* 1986). The transplant success and growth of *Robus idaeus* plantlets due to AMF inoculation was also reported by Pierick (1987). Mycorrhizal establishment significantly improved establishment, growth and mineral nutrition of oil palm plantlets (Blal and Gianinazzi – Pearson, 1988). The effect of AMF inoculation in micropropagated *Populus deltoides* 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 colonisation was obtained in terms of active root growth (Adholeya and Cheema, 1990).

Micropropagated jack plantlets showed increased growth, survival percentage 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 *G. mosseae* or *G. monosporum* enhanced 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 be 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 *G. mosseae* and *G. geosporum* resulted in greater fresh and dry weights of shoots and higher P and K content (Declerck *et al.,* 1994). *In vitro* propagated cherry plants showed better growth and biomass production due to inoculation with AMF (Lovato *et al.,* 1994).

Sivaprasad *et al.* (1995a) observed enhanced survival rate, growth and phenol activity in AMF inoculated jackfruit plantlets. Inoculation with *G. 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.,* 1997). *Glomus constrictum* significantly improved the biomass production, nutrient uptake and acclimatisation of *in vitro* plantlets of *Zizyphus mauritiana* into the field (Mathur and Vyas, 1999). Banana plantlets inoculated with mycorrhiza 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 *G. etunicatum* on micropropagated *Baptisia tinctoria* 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 commercial plant production economically feasible (Grotkas *et al.,* 2000). Micropropagated *Allium sativum* plantlets inoculated with *G. mosseae* exhibited better growth than control plantlets under *ex vitro* conditions (Lubraco *et al.,* 2000). *Glomus fasciculatum, G. erunicatum* and a native *Glomus* sp. inoculation on tissue culture plantlets of banana showed that AM colonisation significantly increased the establishment rate, growth, vigour and biomass production during acclimatisation and after transplanting to pots along with remarkable increase in P and Zn content due to *G. fasciculatum* colonisation (Sivaprasad *et al.,* 1999a).

A significant increase in growth, vigour and biomass production during acclimatisation 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 increased in N and P content in daughter plants (Alarcon *et al.,* 2000).

Inoculation with *G. manihotis* inoculation significantly increased plant growth, root growth and nutrition of micropropagated cassava plants and also increased the field adaptability of plants, especially in marginal soils (Calderon *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 stress 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 *G. clarum* increased plant height, leaf pair number and also exhibited significant difference in enzymatic activities than un inoculated plants (Fernandez *et al.,* 2002). Cultivars of micropropagated banana showed high relative mycorrhizal dependency (RMD) values and also high N, P and K contents (Jaizme and Azcon, 1991).

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 Min *et al.,* 2002). Zeleznik *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* acclimatisation shock (Lata *et al.,* 2003). *Gigaspora margarita* promoted growth, mineral nutrition and

mycorrhizal colonisation of micropropagated banana plants in different stages of rooting (Lins *et al.,* 2003).

Micropropagated seedlings of banana when inoculated with *Gigaspora margarita* in the acclimatisation phase resulted in high mycorrhizal colonisation 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). Biomass production of micropropagated oregano was induced by inoculation with the fungus *Glomus viscosum* (Fortunato and Avato, 2008) and they reported AMF greatly increased parameters such as plant leaf area, fresh and dry weight, number of spicasters and verticillasters in infected plants. Surendra-Gopal *et al.***,** (2009) reported, *Glomus* sp was found to be most effective in enhancing the growth and establishment of micropropagated ginger.

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "*Agrobacterium rhizogenes* mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L." was carried out at the Centre for Plant Biotechnology and Molecular Biology of the College of Horticulture, Vellanikkara. The materials used and methods followed are described below:-

3. 1 Source of seeds

Artemisia annua seeds were obtained from Department of Horticulture, University of Agricultural Sciences, Bangalore for *in vitro* regeneration and *in vitro* shoot multiplication.

3. 2 AMF culture

A standard culture of *Glomus intraradices* was obtained from Department of Agricultural Microbiology, University of Agricultural Sciences, Bangalore.

3. 2. 1 Mass multiplication of *Glomus intraradices* inoculum by pot culture method

The seeds of *Zea mays* (Maize) and *Artemisia annua* (Artemisia) were surface sterilised separately with mercuric chloride (0.1 per cent) for 10 minutes (Maize) and 45 seconds (Artemisia) followed by repeated washing with sterile water. The sterilised soil : sand (2:1) mixture was filled in plastic pots (5 kg). A small quantity of mixture was taken out at the centre with a surface sterilised spatula and the *Glomus intraradices* inoculum 20 g was added to potting mixture. Surface sterilised maize and Artemisia seeds were sown. The plants were watered daily using sterile distilled water. Ruakara nutrient solution (Smith *et al.,* 1983) (Appendix 1) was applied @ 50 ml pot^{-1} at 10 d interval for a period of 90 d. After 90 d the shoot portion of the maize plants were cut and removed. The roots were cut into small pieces and mixed thoroughly

with the soil and sand mixture. The infected root bits, hyphae and chlamydospores of AMF from the pots were used as inoculum for further studies.

3. 2. 2 Per cent root colonisation and spore count

The AMF per cent root colonisation 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 cut into bits of one cm length and then fixed in formalin : acetic acid : alcohol (FAA) (Appendix 2). The root bits fixed in FAA were washed thoroughly in water to remove fixative. The washed root bits were softened by simmering in 10 per cent KOH solution at 90ºC for 10-15 minutes in water bath. After cooling, the excess KOH was washed - off in tap water and then neutralised with two per cent HCl. The root bits were then stained with 0.05 per cent tryphan blue in lactophenol (Appendix 3) for two minutes. The excess stain from the root tissue was removed by cleaning in lactophenol. The root bits were examined under compound microscope (10x) for AMF colonisation. The per cent AMF colonisation was determined using the following formula.

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

3. 2. 3 Isolation of *Glomus intraradices* spore

The AM fungal spores were isolated by wet sieving and decanting method (Gerdemann and Nicolson, 1963). About 250 g of rhizosphere soil was suspended in 1000 ml water and stirred well. After settling of the heavier particles, the supernatant was filtered through a set of sieves of different size (45, 105, 250 and 425 microns). Finally, the soil suspensions present in 45 µ sieve were transferred to 100 ml beaker by gentle washing. The spore suspension was filtered through Whatman No: 1 filter paper. The filter paper containing spores was placed on a Petri dish and observed for

Glomus intraradices spores and transferred to another moistened filter paper under stereomicroscope for further studies.

3. 3 Culture media

3. 3. 1 Chemicals

The major and minor nutrients for the preparation of media were of analytical grade and procured from Merck, Mumbai. Cellulase enzyme was procured from M/s Sisco Research Laboratory (SRL), Mumbai. The primers and enzymes were obtained from M/s Bangalore Genei, Bangalore.

3. 3. 2 Composition of media

Murashige and Skoog (1962) medium was used as basal medium (Appendix. 4) for direct shoot multiplication from the nodal segment of *A. annua*.

3. 3. 3 Preparation of tissue culture medium

Preparation of plant tissue culture media was done as described by Gamborg and Shyluk (1981). 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 colored bottles. A clean steel vessel, rinsed with distilled water was used. Aliquots from all stock solutions were pipetted in proportionate volumes in to 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 was added to it and later, required quantities of sucrose and inositol were added and dissolved. The desired volume was made up by adding distilled water. The pH of the medium was adjusted to 5.7 using 0.1 N 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 and were plugged with absorbent cotton. For solid

media, test tubes (15 cm × 2.5 cm and 10 cm × 2.5 cm) were used whereas for liquid MS jam bottles (200 ml) were used. 15 ml medium was poured in test tube and 50 ml medium in jam bottles. Vessels containing media were sterilised in an autoclave at 121^0C in 15 psi for 20 minutes. The medium was allowed to cool at room temperature and stored in culture room until used.

3. 3. 4 Growth regulators

Cytokinin (0.5 BA) was added in the media for direct shoot multiplication.

3. 3. 5 Carbon source

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

3. 3. 6 Preparation of Yeast Extract Agar (YEA) medium for *Agrobacterium* strains

Clean steel vessels, rinsed with distilled water were used to prepare the media. The ingredients were weighed and 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 7.2 by adding 0.1 N NaOH. For solid media, agar was added at the rate of 20 g l^{-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 121^0C in 15 psi for 20 min. to sterilise the medium and they were further kept in the culture room until used.

3. 4 Culture room

An explant inoculated tube was incubated at 26 ± 2^0C in an air - conditioned culture room with 16 h light photoperiod (1000 lux) from fluorescent tubes. Humidity in the culture room varied from 60 to 80 per cent.

3. 5 *In vivo* regeneration of *Artemisia annua*

About sixty grams of *Artemisia annua* seeds were sown in potting mixture containing soil and sand (2:1) mixture. Ten days after germination, they were transferred into polybags with soil : sand : FYM (1:1:1) mixture and watered daily. These plants were maintained in the glass house, Department of Plant Pathology, as a source of nodal explants for *in vitro* shoot multiplication.

3. 5. 1 Collection and preparation of explant

Nodal segments of *A. annua* plants were used for to produce *in vitro* shoot multiplication (Shaneeja, 2007). Explants were washed in tap water to remove adhering soil particles. The explant was put in soap solution for 10 min. and washed with distilled water to remove detergent. The explant was treated with Bavistin (0.1, 0.15, and 0.2, per cent) for seven minutes. It was then thoroughly washed with distilled sterile water and dried on a sterile blotting paper. The explant was then wiped with 50 per cent ethanol. Further surface sterilisation was carried out under laminar airflow cabinet.

3. 5. 2 Surface sterilisation of explants

Surface sterilisation of explant was carried out in order to make the explant free of contaminants. Surface sterilisation was carried out under aseptic conditions in laminar airflow cabinet. The explant was sterilised with 0.1 per cent mercuric chloride ($HgCl_2$) for 40 seconds. After surface sterilisation, nodal explant was washed 4 times using sterlised water to make sure that explant was free of mercuric chloride. The explant was dried by transferring them onto filter paper pieces on sterile steel plates. Observations were recorded on per cent survival of nodal explants from five to 20 d. For this, hormone free MS basal medium containing 3 per cent sucrose was used. Each treatment had 25 replications.

3. 5. 3 *In vitro* culture establishment and shoot multiplication

Based on survival per cent, the *in vitro* shoots of *Artemisia annua* was multiplied using full MS + 0.5 BA media. These explant were maintained by sub - culturing once in three weeks and leaf used as a source of explant for hairy root induction.

3. 6 *Agrobacterium rhizogenes* mediated genetic transformation

Two strains of *Agrobacterium rhizogenes* MTCC 2364 and MTCC 532 of agropine family obtained from the Institute of Microbial Technology, Chandigarh were used. The protocol for hairy root induction in Artemisia standardised by Shaneeja (2007) was followed in further studies.

3. 6. 1 Culturing of *A. rhizogenes* strains

Agrobacterium rhizogenes MTCC 532 and MTCC 2364 strains were cultured in Yeast Extract Agar (YEA) media (Appendix 5). The solid media was melted and cooled to 40^{o}C and poured into sterilised Petri plates. Each strain was streaked on plates containing YEA media and incubated at 28^{0}C. The growth of bacterial colonies and its characters were observed after 48 h incubation. YEA liquid suspension culture were prepared from single isolated colonies, for infecting the leaf tissues.

3. 6. 2 Pre - culturing of explant

The leaf explant was taken from *in vitro* derived shoots. The explant was cultured on MS solid medium in Petri plates for two days prior to their infection with bacterial suspension culture.

3. 6. 3 Wounding of explant

Wounds were made on the pre - cultured leaf explant using a sterile needle (Stachel *et al.,* 1985). The leaf margins were cut on all sides with a blade and about ten pricks were made on each segment using the needle.

3. 6. 4 Suspension culture inoculation method

The *Agrobacterium* suspension prepared ($O.D_{600}$ ~ 1.0) was transferred in to sterile petri plates. The wounded explant was immersed in the suspension for 15 min. with intermittent gentle agitation. The explant were then blotted dry using sterile blotting paper and placed on solid MS medium without growth regulators taken in the Petri plates. Un - infected explant were placed in the same media and conditions to serve as control.

3. 6. 5 Co - cultivation of explants with *A. rhizogenes*

The infected leaf explant were co - cultured in diffused light at 26 ± 2^0C for three days.

3. 6. 6 Culture media and conditions for hairy root induction

The infected leaf explant after co - cultivation was washed thoroughly with MS liquid medium containing 500 mg l^{-1} cefotaxime (Shaneeja, 2007). After washing, the explant was blotted dry using sterile blotting paper. The explant was transferred to solid MS medium containing 500 mg l^{-1} cefotaxime. They were further cultured at 26 ± 2^0C under diffused light. The number of hairy roots per transformed explant, the transformation percentage and the number of normal roots per explant were recorded ten days after infection.

3. 6. 7 Efficiency of strains in inducing hairy roots

To study the efficiency of strains in inducing hairy roots, the two strains MTCC 2364 and MTCC 532 were inoculated using suspension culture method and co-cultured. Transformation percentage obtained by using each strain in explants was determined. The number of days taken for induction of roots in infected explants by different strains of MTCC 2364 and MTCC 532 were also recorded.

3. 6. 8. Establishment of hairy root cultures

The adventitious roots emerged from the explants within 1 to 4 weeks after infection and those having hairy nature and diffused geotropism were excised out using a sterile blade. The roots were washed in liquid medium containing 250 mg l^{-1} of cefotaxime and blotted dry (Shaneeja, 2007). The individual root tips were separated and transferred to MS solid medium containing 250 mg l^{-1} cefotaxime. The roots were incubated in the culture room at 26 ± 2^0C under diffused light.

3. 7 Inoculation of *Glomus intraradices* in the hairy roots of *Artemisia annua*

3. 7. 1 Isolation of *Glomus intraradices* spore

The spore isolation was done as described in the section 3. 2. 3

3. 7. 2 Surface sterilisation of spore

For surface sterilisation of *G. intraradices* spores, a Whatman No:1 filter paper was placed in the glass funnel with rubber cork and cotton at the tip. This assembly was placed on a conical flask (500 ml). The spores were transferred in the glass funnel containing filter paper and 2 per cent chloramine – T was added for 20 min. followed by 0.2 per cent streptomycin sulphate solution for 10 – 15 min. After surface sterilisation, the antibiotics were drained – off through the funnel and repeatedly washed with sterile water to remove the residues of antibiotics.

3. 7. 3 Germination of *Glomus intraradices* spore

As the AMF spores sometimes remain dormant, the surface sterilised spores were transferred to the Petri plates containing plain bacto agar supplemented with 0.16 mg l^{-1} of cellulase enzyme (Doss *et al.,* 2008) for germination of *G. intraradices* spores. Petri plates with spores were incubated at 25 ± 2^0C for 15 days. Observations were recorded every two days interval on the germination of spores.

3. 8. 4 AMF inoculation of hairy roots in different media conditions

3. 8. 4. 1 Solid medium

Hairy roots were washed using cefotaxime (250 mg l^{-1}) and kept in sterile tissue paper to drain the excess solution. Then hairy roots were placed on MS basal media (without hormone) and 50 germinated spores of *G. intraradices* were placed with on 25 single hairy root under microscope (40x). Plate containing hairy roots of *A. annua* without spore served as control. These plates were kept in 12 - 16 h light period upto 30 d for infection in hairy root.

3. 8. 4. 2 Liquid medium

Hairy roots were washed using cefotaxime 250 mg l^{-1} and kept in sterile tissue paper for draining excess solution. Then 25 single hairy root were transferred to jam bottle, which contained 50 ml of MS basal medium. Fifty germinated spores of *G. intraradices* were picked up with endothermic needle under microscope (40x) and transferred to the MS basal media containing hairy roots.

3. 8. 5 Culture conditions

3. 8. 5. 1 Static condition

Solid media containing AMF spores on hairy roots were maintained under static condition at 12 - 16 h light for 30 d. Hairy roots without spore were maintained in the same media and condition to serve as control.

3. 8. 5. 2 Shaking condition

Liquid media containing AMF spores on hairy roots were maintained under shaking condition at 12 - 16 h light for 30 d. Hairy roots without spores were maintained in the same media and condition to serve as control.

3. 9. Molecular detection of *Glomus intraradices* in the hairy roots of *Artemisia annua*

3. 9. 1 Protocol for isolation of DNA

DNA from the infected hairy roots of *A. annua* was isolated after 60 days of inoculation (Bonito *et al.,* 1995).

3. 9. 1. 1 Materials for isolation of DNA

i. Chelex 100 resin (Bio-Rad Lab.)
ii. 1M Tris – HCL

Procedure

- Template DNA for PCR was obtained from crushed root samples by boiling each sample in 800 µl of extraction buffer (1 M Tris – HCL, pH 8.5) for 15 min.
- DNA template from roots were obtained by adding Chelex 100 resin (Bio-Rad Lab.) to achieve 5% final concentration to the extraction buffer prior to boiling.
- The resin was removed by centrifugation at 12, 000 × g for 20 sec. The supernatant was saved to use as DNA sample.
- The processed samples were either immediately used for PCR or frozen at - 20^0C.

3. 9. 2 PCR mixtures

The DNA sample was diluted with equal volume of sterile water and used for assay. Fifty µl of final reaction mixture was used for PCR assay. The other components are

• VANS1 (Forward primer)	50 pmol	1µl
• NS21 (Reverse primer)	50 pmol	1µl
• Deoxynucleoside triphosphate	200 µM each	1µl
• Tris - HCL (pH 8.4)	20 mM	1.25µl
• KCl	100 mM	1µl
• $MgCl_2$	3 mM	1.25µl
• Gelatin	0.02 %	2µl
• *Taq* DNA polymerase	1.25U	2µl
• DNA sample	50ng	1µl
• Sterile distilled water		38.5µl

3. 9. 2. 1 Thermal cycler reactions

▪ Initial denaturation	2 minutes at 94^0C	
▪ Denaturation	60 second at 94^0C	29 times repeated
▪ Annealing	45 second at 50^0C	
▪ Extension	60 second at 72^0C	
▪ Final extension	10 minutes at 72^0C	

3. 9. 4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al.* (1989) to check the quality of DNA from *G. intraradices* infected hairy roots of *A. annua*.

Materials

- Agarose (Bangalore Genei)
- 50X TAE buffer (pH 8.0)
- Electrophoresis unit (Biorad, USA), power pack, casting tray, comb

- 6X loading / tracking dye (Bangalore Genei)
- Ethidium bromide solution (stock 10mg / ml; working concentration, 0.5µg / ml)
- Gel documentation and analysis system (Alpha imager TM 1200)

Procedure

1. 1X TAE buffer was prepared from 50 X TAE stock solution.
2. Agarose 3.0 per cent (Bonito *et al.,* 1995) was weighed and added to 1X TAE. It was boiled till the agarose dissolved completely and then cooled to lukewarm temperature.
3. Ethidium bromide was added to a final concentration of 0.5µg / ml as an intercalating dye of DNA and mixed well.
4. The open ends of the gel casting tray were sealed with a cellophane tape and placed on a perfectly horizontally levelled platform.
5. The comb was placed properly and molten agarose was poured into the tray, allowing it to solidify.
6. After the gel was completely set (15 to 20 minutes at room temperature), the comb and cellophane tape were carefully removed.
7. The gel was placed in the electrophoresis tank with the wells near the cathode and submerged in 1 X TAE to a depth of 1cm.
8. A piece of cellophane tape was pressed on a solid surface and 1µl 6X loading dye was dispensed in small quantity on the tape. A quantity of 10.0 to15.0 µl of PCR product was added to each slot mixed well by pipetting in and out for 2 to 3 times. The mixture was loaded in the wells with the help of micropipette. 100 bp lader (Bangalore Genei) was used as the molecular weight marker.
9. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 70 volts.
10. The power was turned off when the tracking dye reached at about 3cm from the anode end.

3. 9. 5 Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using Alpha Imager TM 1200 documentation and analysis system.

3. 10 Evaluation of different *Glomus intraradices* inoculum for growth and establishment of micropropagated ginger

A pot culture experiment was conducted using soil : sand : farm yard manure (1:1:1) as potting mixture to evaluate the effect of AMF inoculum for the growth and establishment of micropropagated ginger plant. *G. intraradices* mass multiplied under pot culture was used as inoculum. Each plant was inoculated with 30 g of *G. intraradices* inoculum. The treatment details were as follows:

T_1 - *Artemisia annua* based *G. intraradices* inoculum (in sand)

T_2 - *Artemisia annua* based *G. intraradices* inoculum (soil : sand : farm yard manure (1:1:1))

T_3 - Maize based *G. intraradices* inoculum (soil : sand : farm yard manure (1:1:1))

T_4 - Control

The experiment was laid out in Completely Randomised Design (CRD) with ten replications each. Observations on plant biometric characters such as survival rate, plant height, number of leaves, number of tiller / shoots, fresh weight and dry weight of shoots and roots.

3. 10. 1 Survival rate of plants

The number of plants survived were recorded at 15 days interval as per cent survival.

3. 10. 2 Plant height

The height of plant (cm) from the ground level to the growing tip of each plant was measured and recorded at 15 days interval.

3. 10. 3 Number of leaves

The total numbers of fully opened leaves developed per plant were counted.

3. 10. 4 Number of tillers

The total number of tillers developed per plant was counted and the mean value was recorded.

3. 10. 5 Fresh weight of shoot and root

The fresh weight of shoots and roots were recorded separately after uprooting of the plants 75 DAI.

3. 10. 6 Dry weight of shoot and root

The dry weight of shoot and root were recorded after drying to a constant weight at 60ºC.

3. 10. 7 Analysis of the data

The plant biometric characters of micropropagated ginger were analysed by using MSTATC statistical software programme. The output data was obtained through ONEWAY method, through which the mean values of LSD values for number of leaves, plant height and number tillers were determined. The mean values for leaf numbers, plant height and number of tillers were ranked by using Duncan's Multiple Range Test (DMRT) (Panse and Sukhatme, 1978).

Results

4. RESULTS

The results of the study on "*Agrobacterium rhizogenes* mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L." carried out at the Centre for Plant Biotechnology and Molecular Biology of the College of Horticulture, Vellanikkara are presented below.

4. 1 Colonisation of *Glomus intraradices* on Maize and Artemisia

The results on colonisation and spore count of *Glomus intraradices* in maize and *Artemisia annua* are presented in Table 1 and Plate 1 (a, b, c). *Artemisia annua* recorded 15 per cent root colonisation when compared, maize which recorded 60 per cent. The number of spores of *Glomus intraradices* was 38 (per 50 g) in maize inoculum where as it was only 11 spores in *Artemisia annua* inoculum. The per cent root colonisation and spore count of *Glomus intraradices* in *Artemisia annua* was less when compared with maize. Hyphae attached vesicles were noticed in the root section of maize (plate 1 a), artemisia (plate 1 b) and golden yellow coloured tiny spores (plate 1 c) were isolated from the inoculum.

4. 2 Seed germination of *Artemisia annua* under *in vitro* and *in vivo* conditions

In vitro and *in vivo* seed germination of *Artemisia annua* using different media are presented in Table 2. Sterile sand used for *in vitro* seed germination of *Artemisia annua* recorded no germination of seeds. The half strength MS + 1 BA + 1 NAA and full strength MS + 1 BA + 1 NAA combination also did not show *in vitro* seed germination even after 70 days of seed inoculation.

In vivo seed germination of *Artemisia annua* using sand, soil and sand (1:1), sand and vermicompost (1:1) recorded 53 per cent, 78 per cent, 85 per cent respectively.

Table 1. Colonisation of *Glomus intraradices* in maize and artemisia

Sl. No	Host	Root colonisation (%)	AMF Spore count / (50g) inoculum
1	*Zea mays* (Maize)	60	38
2	*Artemisia annua* (Artemisia)	15	11

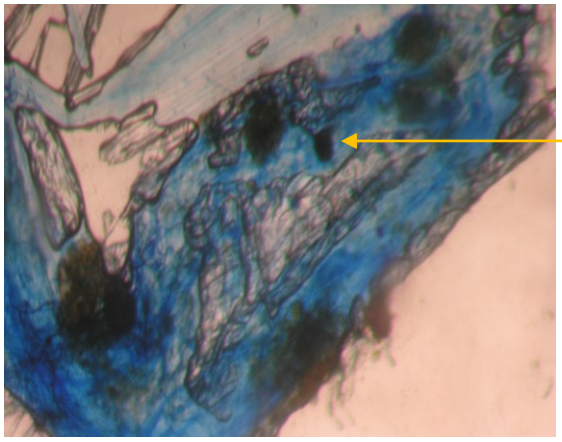
Each value represents an average of five replication

Table 2. Seed germination of *Artemisia annua* under *in vitro* and *in vivo* conditions

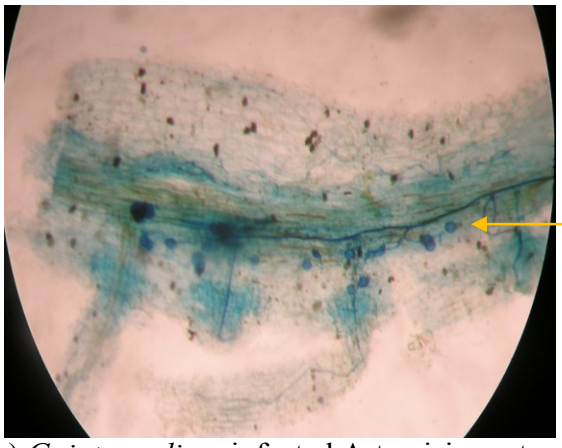
Sl. No	Media	Germination per cent
***In vitro* seed germination**		
1	½ MS + 1BA + 1NAA	0
2	FMS + 1BA + 1NAA	0
3	Sterile sand	0
***In vivo* seed germination (unsterile condition)**		
4	Sand	53
5	Soil and sand (1:1)	78
6	Vermi compost and sand (1:1)	85

Five replication were maintained for each media

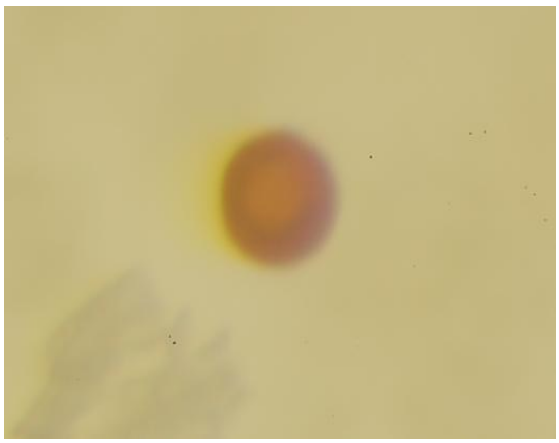
Plate 1: Root colonisation and spore of *Glomus intraradices* in different host



(a) *G. intraradices* infected maize root section



(b) *G. intraradices* infected Artemisia root section



(c) *G. intraradices* spore

4. 3 Surface sterilisation of *Artemisia annua* nodal explant with different concentrations of bavistin

Artemisia annua plants were maintained in the net house (Plate 2) and nodal explant were used to obtain *in vitro* plants for hairy root induction using leaf. In order to prevent contamination, bavistin with different concentrations were used. High percentage of fungal contamination was found at 0.1 Bavistin + 0.1 per cent $HgCl_2$ (31.82 %) followed by 22.73 per cent at 0.15 Bavistin + 0.1 per cent $HgCl_2$ and 13.64 per cent at 0.2 Bavistin + 0.1 per cent $HgCl_2$ (Table 3). Bacterial contamination was recorded in the case of 0.1 per cent bavistin (9.1 per cent). However, no bacterial contamination was noticed at 0.15 per cent and 0.2 per cent Bavistin + 0.1 per cent $HgCl_2$ but tissue was died ten days after inoculation.

4. 4 *In vitro* culture establishment of *Artemisia annua*

Fifty tubes of shoots were multiplied and sub-cultured on Murashige and Skoog (MS) medium with 0.5 BA (Plate 3).

4. 5 Effect of different *A. rhizogenes* strains on hairy root induction of *A. annua* under *in vitro*

In vitro leaf explants of *A. annua* were used for hairy root induction using both MTCC 532 and MTCC 2364 strains of *A. rhizogenes* in order to select the suitable strain for hairy root induction (Plate 4).

Hairy roots were induced for *in vitro* mass multiplication of *G. intraradices*. Creamy coloured, feathery roots and negative geotropic nature of roots were noticed on the seventh day after inoculation on hormone free MS basal media on leaf explants treated with both strains. The number of hairy roots per explant was high (12) in the case of MTCC 532 strain (Table 4 and Plate 5b). The number of leaf explant that responded to hairy root formation was high (18) in the case of MTCC 2364 (Table 4). MTCC 532 strain induced hairy roots recorded fast growth during the proliferation

Table 3. Surface sterilisation of *Artemisia annua* nodal explant with different concentrations of bavistin

Sl. No	Treatments	Survival percentage (10 DAI)	Microbial contamination (%)
1	0.1% Bavistin + 0.1% $HgCl_2$	68.18	(31. 82)
2	0.15 Bavistin + 0.1% $HgCl_2$	4.0	(22. 73)
3	0.2 Bavistin + 0.1% $HgCl_2$	0.0	(13. 64)

Each treatment maintained with 25 tubes

Table 4. Effect of MTCC 532 and MTCC 2364 *A. rhizogenes* strains on hairy root induction of *A. annua* under *in vitro*

Sl. No	Parameters	MTCC 532	MTCC 2364
1	Number of leaf explant per plate	20	20
2	Number of explant responded to hairy roots	4	18
3	Days taken for hairy root formation	7	7
4	Hairy root percentage	20	90
5	Number of hairy roots per explant	12	2

Plate 2: *In vivo* germinated *Artemisia annua* L. seedlings and its maintenance

Young seedling stage

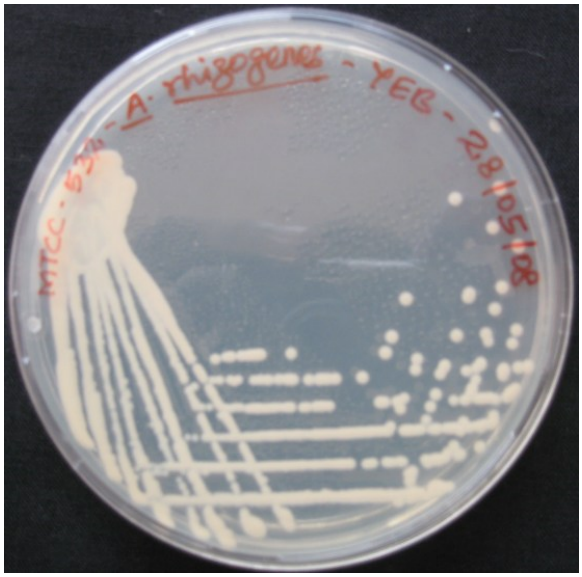
Flowering stage

Different stages of *Artemisia*

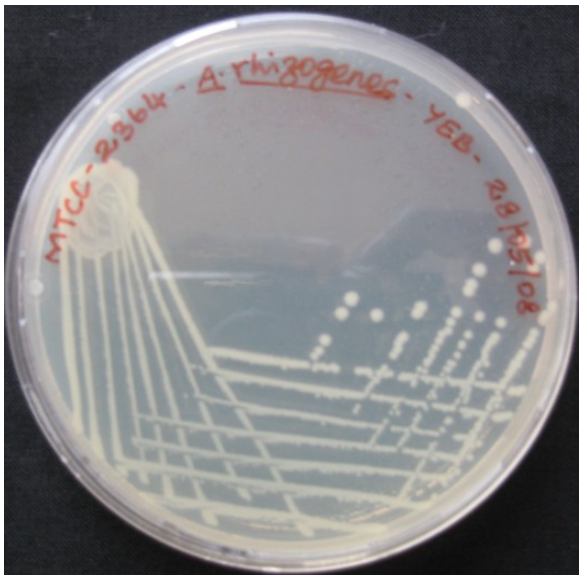
Artemisia seedling maintenance

Plate 3: Shoot multiplication of *A. annua* from nodal explant

Plate 4: *Agrobacterium rhizogenes* strains used for hairy root induction



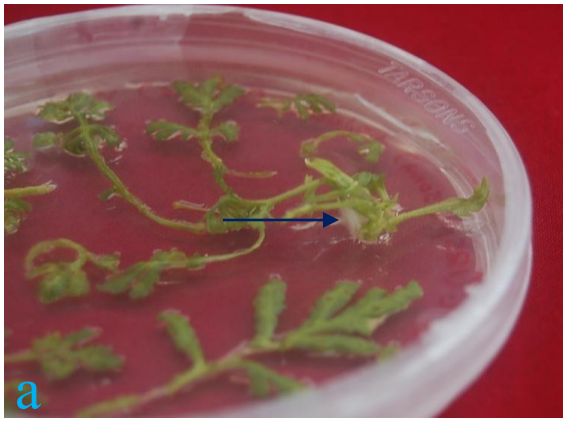
MTCC 532



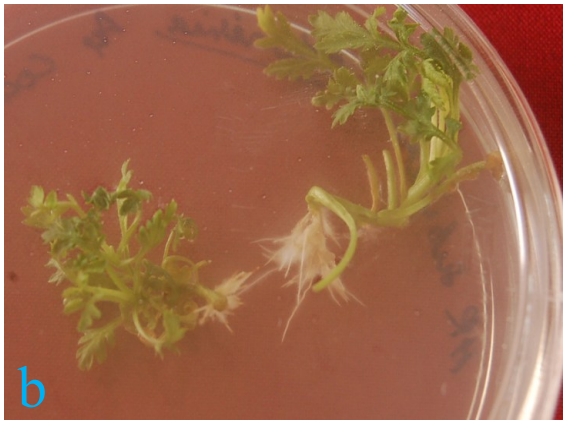
MTCC 2364

Plate 5: Effect of different *A. rhizogenes* strains on hairy root induction (Arrow indicates) of *A. annua* under *in vitro*

MTCC 532



MTCC 532



7th day after co – cultivation

MTCC 532

14th day after co - cultivation

MTCC 532

30th day after co - cultivation

period. Due to this fast growth and larger number of hairy roots per explant, MTCC 532 was selected for hairy root induction in *A. annua*.

4. 6 Effect of cellulase enzyme on spore germination of *Glomus intraradices* under *in vitro* condition

Glomus intraradices spores were surface sterilised and germinated on water agar media supplemented with 16.0 mg l^{-1} cellulase enzyme for inoculation of hairy roots. Spore wall of AMF were broken on 5^{th} day of spore inoculation. Out of 185 spores used for germination, 32 spores germinated (17.3 %) (Table 5) where as in the case of control only 9 spores germinated (4.86 %).

4. 7 Infection of *G. intraradices* in hairy roots of *A. annua* on solid media under *in vitro*

The root infection by *G. intraradices* in *A. annua* hairy roots in solid MS basal media indicated no infection in the hairy roots and the hairy roots were contaminated with fungus. The hairy roots showed poor growth or no growth on the media (Table 6) (Plate 6a, 6b).

4. 8 Infection of *G. intraradices* from hairy roots of *A. annua* in liquid media (static) under *in vitro* condition

In liquid MS basal media under static condition, no growth of hairy root was noticed. The bacterial and fungal contaminants were noticed in the media (Table 7) (Plate 7a, 7b).

4. 9 Infection of *G. intraradices* from hairy roots of *A. annua* in liquid media (shaking) under *in vitro* condition

In liquid MS basal media under shaking condition, germinated spores of *G. intraradices* placed on the hairy roots showed no root infection due to poor growth of hairy roots and bacterial contamination (Table 8) (Plate 8a, 8b).

Table 5. Effect of cellulase enzyme on spore germination of *Glomus intraradices* under *in vitro* condition

Sl. No	Parameters	Control	Cellulase (16.0 mg l^{-1})
1	Total number of spores placed on the water agar media	185	185
2	Total number of spores germinated	9	32
3	Germination per cent	4.86	17.3

Table 6. Infection of *G. intraradices* on hairy roots of *A. annua* under *in vitro* (solid media)

Sl. No	Parameters	Observation
1	Number of single hairy roots inoculated	25
2	Number of sample survived after 3 weeks	12
3	Number of hairy roots proliferated	Nil
4	Number of hairy roots contaminated	13
5	Per cent number of hairy roots infected with AMF	Nil

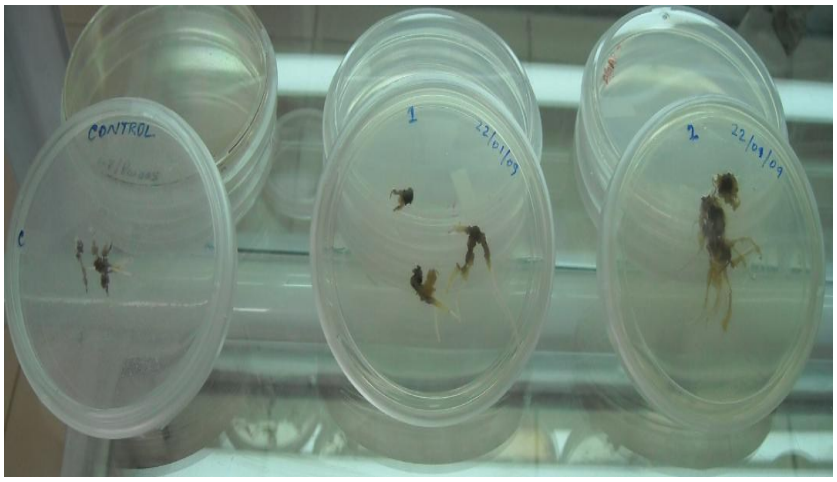
Table 7. Infection of *G. intraradices* on hairy roots of *A. annua* under *in vitro* (liquid media and static condition)

Sl. No	Parameters	Observation
1	Number of single hairy roots inoculated	25
2	Number of sample survived after 3 weeks	7
3	Number of hairy roots proliferated	Nil
4	Number of hairy roots contaminated	18
5	Per cent number of hairy roots infected with AMF	Nil

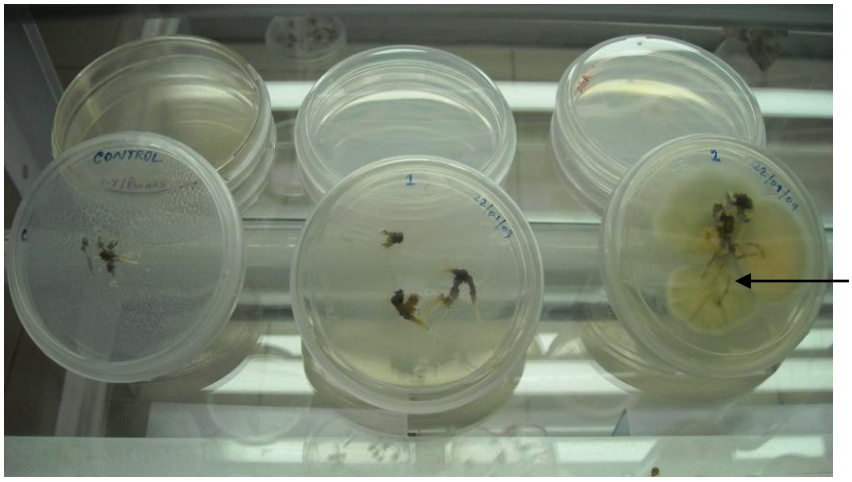
Table 8. Infection of *G. intraradices* on hairy roots of *A. annua* under *in vitro* (liquid media and shaking condition)

Sl. No	Parameters	Observation
1	Number of single hairy roots inoculated	25
2	Number of sample survived after 3 weeks	5
3	Number of hairy roots proliferated	Nil
4	Number of hairy roots contaminated	20
5	Per cent number of hairy roots infected with AMF	Nil

Plate 6: *G. intraradices* inoculated hairy roots of *A. annua* on solid media under *in vitro* condition



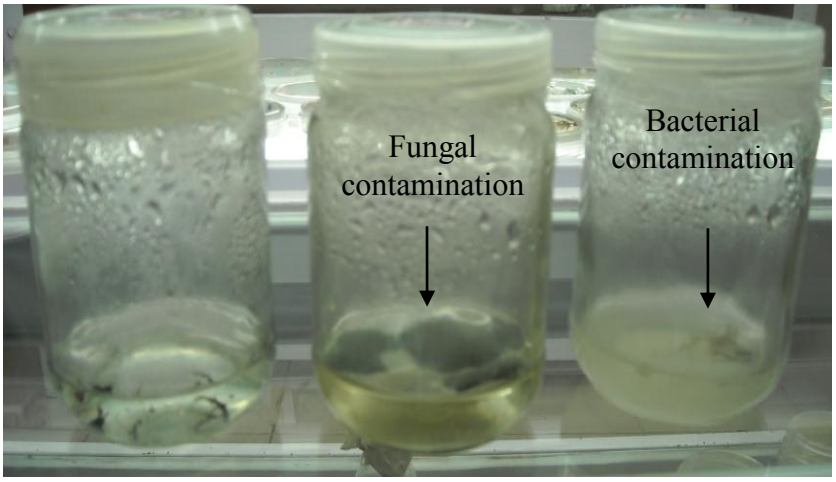
(a) Solid MS basal media (Static)



(b) Fungal contamination

Plate 7: *G. intraradices* inoculated hairy roots of *A. annua* in liquid media (static) under *in vitro* condition

(a) Liquid MS basal media (Static)



(b) Fungal and bacterial contamination

Plate 8: *G. intraradices* inoculated hairy roots of *A. annua* in liquid media (shaking) under *in vitro* condition

(a) Liquid MS basal media (Shaking)

(b) Bacterial contamination

4.10 Molecular detection of *Glomus intraradices* in the hairy roots of *Artemisia annua*

Polymerase chain reaction was done with DNA of *Glomus intraradices* multiplied in hairy roots of *Artemisia annua* by using universal primers VANS 1 and NS 21. There was no amplification found and it confirmed no *G. intraradices* infection in the hairy roots (Plate 9).

4. 11 Effect of different *Glomus intraradices* inoculum on survival percentage of micropropagated ginger under pot culture

Glomus intraradices multiplied in the roots of *Artemisia annua* and maize were tested for its efficiency in enhancing the growth and establishment of micropropagated ginger under pot culture. The maximum survival percentage (100 %) was recorded in the case of *A. annua* based inoculum (T_2) followed by maize based inoculum (T_3) (Table 9). However, *A. annua* based inoculum in sand (T_1) recorded 80 % survival of the micropropagated ginger. Control plants (T_4) recorded only 40 per cent survival.

4. 12 Effect of different *G. intraradices* inoculum on number of leaves

Treatments varied significantly with respect to number of leaves (Table 10). *A. annua* based inoculum (T_2) recorded maximum number of leaves (7.8) followed by maize based inoculum (T_3 - 6.5) and *A. annua* based inoculum in sand (T_1 - 5.9). Control plants (T_4) recorded least number of leaves (4.7) at the 15 DAI (Days After Inoculation of spores).

At 75 DAI, maximum number of leaves were recorded in T_2 (*A. annua* based inoculum) (21.0) followed by T_3 (maize based inoculum) with 17.0 and T_1 (*A. annua* based inoculum in sand) (10.8). The least number of leaves were recorded in control plants (2.8).

Table 9. Effect of different *Glomus intraradices* inoculum on survival percentage of micropropagated ginger under pot culture

Sl. No	Treatments	Survival percentage (DAI)				
		15	30	45	60	75
1	T_1 (*A. annua* based inoculum) (in sand)	100	90	90	80	80
2	T_2 (*A. annua* based inoculum)	100	100	100	100	100
3	T_3 (Maize based inoculum)	100	100	90	90	90
4	T_4 (Control)	100	60	40	40	40

T_1 - Sand as substrate; T_2 - T_4 – Potting mixture containing Soil : Sand : Farm Yard Manure (1:1:1) Each value represents an average of 10 replication.

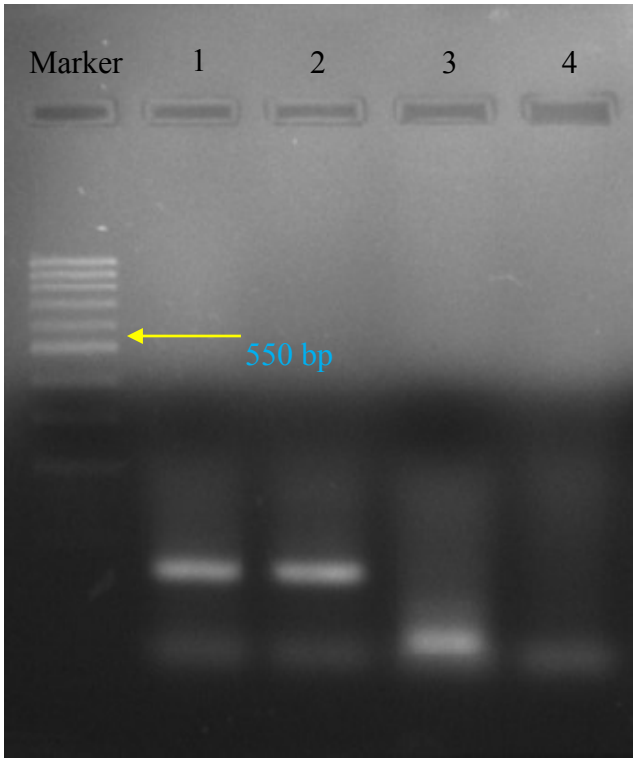
DAI - Days After Inoculation of spores

Table 10. Effect of different *G. intraradices* inoculum on number of leaves in micropropagated ginger under pot culture

Sl. No	Treatments	Number of leaves (15 Days Interval)				
		15	30	45	60	75
1	T_1 (*A. annua* based inoculum in sand)	5.9^{ab}	6.5^{b}	8.7^{a}	10.2^{b}	10.8^{b}
2	T_2 (*A. annua* based inoculum)	7.8^{a}	9.3^{a}	11.7^{a}	16.5^{a}	21.0^{a}
3	T_3 (Maize based inoculum)	$6.5a^{b}$	7.5^{ab}	9.4^{a}	13.1^{ab}	17.0^{a}
4	T_4 (Control)	4.7^{b}	1.4^{c}	1.0^{b}	1.9^{c}	2.8^{c}
5	LSD value	1.895	2.454	3.409	4.832	6.165

Each value represents an average of 10 replication

Plate 9: Molecular detection of *Glomus intraradices* in the hairy roots of *Artemisia annua*



1 and 2 - Control positive - *Glomus intraradices* infected hairy roots of *Artemisia annua*
3 and 4 - Control negative - *Glomus intraradices* infected hairy roots of Maize

4. 13 Effect of different *G. intraradices* inoculum on plant height

Treatments varied significantly with respect to plant height (Table 11). The T_3 (maize based inoculum) recorded maximum plant height (8.54 cm) followed by T_1 (*A. annua* based inoculum in sand) (8.34 cm) and T_2 (*A. annua* based inoculum) (7.93 cm). Control plants (T_4) recorded least plant height (6.07 cm) at 15 DAI.

At 75 DAI, maximum plant height was recorded in T_2 (*A. annua* based inoculum) (28.32 cm) followed by T_3 (maize based inoculum) with 24.42 cm and T_1 (*A. annua* based inoculum in sand) (18.56 cm). The least plant height was recorded in control plants (5.98 cm).

4. 14 Effect of different *G. intraradices* inoculum on number of tillers

Treatments varied significantly with respect to number of tillers (Table 12). T_2 (*A. annua* based inoculum) recorded maximum tillers (1.1) followed by T_3 (maize based inoculum) (0.6). T_1 (*A. annua* based inoculum in sand) (0.0) and control plants (T_4) recorded no tillers.

Maximum number of tillers were recorded in T_2 (*A. annua* based inoculum) (2.4) followed by T_3 (maize based inoculum) with 1.6 and T_1 (*A. annua* based inoculum in sand) (0.6) at 60 DAI. The least number of tillers (0.1) was recorded in control plants.

4. 15 Effect of different *G. intraradices* inoculum on fresh and dry weight of micropropagated ginger under pot culture

4. 15. 1 Fresh shoot weight

The treatments differed significantly for fresh shoot biomass of micropropagated ginger. Maximum shoot fresh weight was recorded in T_2 (*A. annua* based inoculum) (6.79 g) followed by T_3 (maize based inoculum) with 5.72 g and T_1

Table 11. Effect of different *G. intraradices* inoculum on plant height of micropropagated ginger under pot culture

Sl. No	Treatments	Plant height (cm) (15 Days after Planting / Interval)				
		15	30	45	60	75
1	T_1 (*A. annua* based inoculum in sand)	8.34[a]	7.69[a]	12.39[a]	15.94[a]	18.56[b]
2	T_2 (*A. annua* based inoculum)	7.93[a]	9.3[a]	13.78[a]	21.14[a]	28.32[a]
3	T_3 (Maize based inoculum)	8.54[a]	8.9[a]	11.55[a]	18.88[a]	24.42[ab]
4	T_4 (Control)	6.07[a]	2.87[b]	1.68[b]	4.13[b]	5.98[c]
5	LSD value	2.41	2.59	3.97	6.20	7.41

Each value represents an average of 10 replication

Table 12. Effect of different *G. intraradices* inoculum on number of tillers of micropropagated ginger under pot culture

Sl. No	Treatments	Number of tillers (15 Days After Planting)			
		30	45	60	75
1	T_1 (*A. annua* based inoculum in sand)	0.0[b]	0.2[bc]	0.4[b]	0.6[b]
2	T_2 (*A. annua* based inoculum)	1.1[a]	1.1[a]	1.2[a]	2.4[a]
3	T_3 (Maize based inoculum)	0.6a[b]	0.8a[b]	1.1[a]	1.6[a]
4	T_4 (Control)	0.0[b]	0.0[c]	0.0[b]	0.1[b]
5	LSD value	0.67	0.61	0.58	0.83

(*A. annua* based inoculum in sand) (2.24 g). The least shoot fresh weight was recorded in control plants (0.72 g) at 75 DAI (Table 13).

4. 15. 2 Fresh root weight

The treatments differed significantly in the case of root fresh weight. Maximum root fresh weight was recorded in T_2 (*A. annua* based inoculum) (2.84 g) followed by T_3 (maize based inoculum) with 2.56 g and T_1 (*A. annua* based inoculum in sand) (1.67 g) (Table 13). The least root fresh weight was recorded in control plants (0.27 g) at 75 DAI.

4. 15. 3 Dry shoot weight

The treatments were differed significantly for biomass of micropropagated ginger in the case of shoot dry weight (Table 13). Maximum shoot dry weight were recorded in T_2 (*A. annua* based inoculum) (0.77 g) followed by T_3 (maize based inoculum) with 0.63 g and T_1 (*A. annua* based inoculum in sand) (0.29 g). The least shoot dry weight was recorded in control plants (0.07 g) at 75 DAI.

4. 15. 4 Dry root weight

The treatments differed significantly in the case of root dry weight. Maximum root dry weight were recorded in T_2 (*A. annua* based inoculum) (0.19 g) followed by T_3 (maize based inoculum) with 0.17 g and T_1 (*A. annua* based inoculum in sand) (0.14 g). The least root dry weight was recorded in control plants (0.02 g) at 75 DAI.

Table 13. Effect of different *G. intraradices* inoculum on fresh and dry weight of micropropagated ginger under pot culture (75 DAI)

Sl. No	Treatments	Fresh weight (g) / Plant		Dry weight (g) / Plant		Plant fresh weight (g) / Plant	Plant dry weight (g) / Plant
		Shoot	Root	Shoot	Root		
1	T_1 (*A. annua* based inoculum in sand)	2.24^{b}	1.67^{b}	0.29^{b}	0.14^{a}	3.91	0.44
2	T_2 (*A. annua* based inoculum)	6.79^{a}	2.84^{a}	0.77^{a}	0.19^{a}	9.63	0.97
3	T_3 (Maize based inoculum)	5.72^{a}	2.56^{ab}	0.63^{a}	0.17^{a}	8.28	0.80
4	T_4 (Control)	0.72^{b}	0.27^{c}	0.08^{b}	0.02^{b}	0.99	0.09
5	LSD value	2.13	0.99	0.24	0.07	1.12	0.31

Each value represents an average of 10 replication

4. 16 Effect of different *G. intraradices* inoculum on fresh and dry weight of micropropagated ginger plants under pot culture

4. 16. 1 Plant fresh weight

The treatments differed significantly for fresh biomass of micropropagated ginger (Table 13). Maximum fresh plant weight was recorded in T_2 (*A. annua* based inoculum) (9.63g) followed by T_3 (maize based inoculum) with 8.28 g and T_1 (*A. annua* based inoculum in sand) (3.91 g). The least fresh weight was recorded in control plants (0.99 g) at 75 DAI.

4. 16. 2 Plant dry weight

The treatments differed significantly for dry biomass of micropropagated ginger (Table 13). Maximum dry plant weight was recorded in T_2 (*A. annua* based inoculum) (0.97 g) followed by T_3 (maize based inoculum) with 0.80 g and T_1 (*A. annua* based inoculum in sand) (0.44 g). The least dry weight was recorded in control plants (0.09 g) at 75 DAI.

Plate 10: Different *G. intraradices* inoculum for growth and establishment of micropropagated ginger under pot culture studies (75 DAI)

Plate 10 a - T_1 *A. annua* based *G. intraradices* inoculum (in sand) treated plant
Plate 10 b - T_2 *A. annua* based *G. intraradices* inoculum treated plant
Plate 10 c - T_3 Maize based *G. intraradices* inoculum treated plant
Plate 10 d - T_4 Control plant
Plate 10 e - one of the control plant dead during establishment

Plate 11: *G. intraradices* inoculated micropropagated ginger under pot culture

All the four treatments completely randomised

T_1 *A. annua* based *G. intraradices* inoculum in sand
T_2 *A. annua* based *G. intraradices* inoculum in potting mixture
T_3 Maize based *G. intraradices* inoculum in potting mixture
T_4 Control plant

Discussion

5. DISCUSSION

Arbuscular mycorrhizal fungi are beneficial symbiotic microorganisms that colonise the roots and increase the growth and yield of most of the crops. The improved plant growth is attributed to increased nutrient uptake, production of growth promoting substances, tolerance to drought, salinity and transplantation shock, resistance to plant pathogens and synergistic interaction with beneficial soil microorganisms. AM fungi are obligate symbionts which cannot be cultured in laboratory media. Even though several methods have been employed to culture these fungi on artificial media, it has not become successful. This is an important lacuna for successful large scale adoption under sustainable farming system. At present, the AMF is mass multiplied using a suitable plant host under pot culture. However, the method is time consuming and cumbersome and there is also a high possibility of cross contamination during mass multiplication.

Therefore, an attempt was made in the present study to mass multiply *Glomus intraradices* in the *Agrobacterium rhizogenes* mediated hairy roots in *Artemisia annua*. The root organ culture of AMF provides pure, viable and contamination free inoculum in a smaller space and is therefore more efficient than the conventional mode of pot culture multiplication (Tiwari and Adholeya, 2002). *A. rhizogenes* is a soil borne pathogen inducing hairy roots in dicotyledonous plants. When wounded tissues are inoculated with *A. rhizogenes*, hairy roots are formed. These hairy roots grow faster and are produced in large numbers. Hence, these hairy root biomass can be an ideal substrate for the mass multiplication of AMF. In the present study, *A. annua* was used for the production of hairy roots as the protocol for the hairy root production has already been standardised (Shaneeja, 2007).

In a comparison on the per cent root infection and spore count of *G. intraradices* in maize and *A. annua*, the former indicated high per cent root infection (60%) and spore count (38 nos). The per cent mycorrhizal root colonisation varies

from plant to plant species and the distribution and abundance of VA mycorrhizal fungi depends on plant species as well as soil type (Eranna *et al.,* 1998). The maize plant grows very fast and produces abundant roots for AMF infection. In the case of *A. annua*, the per cent root colonisation was 15 per cent with spore count of 11 / 50 g of inoculum. Not all AM fungi are equally capable of colonising roots with spores and hyphae in root fragments (John and Miranda, 2002). The *G. intraradices* mass multiplied using *A. annua* under sterile conditions had less root colonisation and spore count.

The most important characteristics of hairy roots required for the mass production of *G. intraradices* / AMF is rapid growth and high stability. However, there is a difference in growth of hairy roots among different species and between different root clones of the same species (Sevon and Oksman - Caldentey, 2002).

A. rhizogenes was selected for hairy root induction of *A. annua*. The results indicated maximum numbers of hairy roots/explant (12) in the case of MTCC 532 strain when compared with MTCC 2364 (2). The *A. rhizogenes* strains are not only influenced by its genetic diversity but also due to culture medium, culture conditions and explants used for induction. Several authors have reported the use of different media (Mano *et al.,* 1986; Kintzions, 2003; Chandran and Potty, 2008). Therefore, MTCC 532 strain was selected for further studies.

For the successful infection of AMF on hairy roots, the AMF spores should be pure and viable. Hence, the AMF spores were surface sterilised and germinated on water agar (1.5%) containing cellulase enzyme 0.16 mg l^{-1}, so as to inoculate only germinated *G. intraradices* spores to *A. annua* hairy roots. The germination percentage of *G. intraradices* recorded was 17.3 per cent on water agar media containing cellulase enzyme when compared with control (4.86 %) indicating the need for inoculation of pre - germinated spores for infection.

The effect of *G. intraradices* on hairy root infection on solid media and static condition revealed no mycorrhizal infection in hairy roots of *A. annua.* Even in liquid media under static and shaking conditions there was no AMF infection. Moreover, the liquid media were contaminated with fungus and bacteria. One of the constraints in the micropropagation of plants and hairy root proliferation is the contamination. The contaminants might interfere with the AMF infection as the AMF is slow in its growth. Doss *et al*. (2008) reported that the success of axenic culturing of transformed root with AM fungi is based on purity of AMF spores used. Sometimes, the impurities associated with AMF are not easily eradicated even after sterilisation (Rai, 2001). Hence, there is a need to adopt combination of sterile mixture of antibacterial and / or antifungal agents to prevent contamination of AMF spore as well as the culture medium used for co - cultivation.

Axenically infected mycorrhizal roots can also be used as inoculum to overcome the problem of contamination (Elmeskaoui *et al.,* 1995; Plenchette *et al.,* 1996; Declerek *et al.,* 2000). In the present study, *in vitro* co - culture of *G. intraradices* on Ri - T - DNA transformed roots of *A. annua* revealed no AMF infection. It is important to select a suitable medium for co – cultivation of AMF and hairy roots. In the present study, the hairy roots of *A. annua* were induced in the MS media. The selection of media is important as certain types contain inhibitory properties (Hepper, 1978). Since, the MS medium contains sodium sulphate, phosphorus and sucrose in the high concentration, a change of medium for AMF infection is essential before tripartite culture stage (Elmeskaoui *et al.,* 1995; Becard and Fortin, 1988). Schubert *et al.* (1987) reported that the spores of AMF failed to germinate on ½ MS medium used for grapevine micropropagation where as the spores germinated readily on water agar (Hepper and Smith, 1976). On MS medium, the spores neither germinated nor established symbiosis. The medium exhibited adverse effects so much that even the pre germinated spores of AMF stopped growth (Rai, 2001). However, co - cultivation of AMF with *A. rhizogenes* mediated

transformed hairy roots of tomato (Doss *et al.,* 2008), carrot (Mugnier and Mosse, 1987) *Medicago truncatula* (Chabaud *et al.,* 2006) and grape vine (Gribaudo *et al.,* 1995) have been successful. In the present study, AMF could not infect the transformed roots of *A. annua.* It has been observed from previous literature that much attention is being given towards the AMF and the suitable host is particularly neglected. Hence, behaviour of the host plant needs to be improved for better colonisation by AMF (Rancillac *et al.,* 1996). The AMF colonisation of roots can also be enhanced by mycorrhizal helper bacteria in soil like *Bacillus mycoides* and *Pseudomonas fluorescens* (Von, 1998). However, sterilisation procedure and inoculation method needs to be perfected.

Since, it was not successful to infect and multiply AMF on the hairy root, further work was carried out with *Artemisia annua* based inoculum mass multiplied under pot culture. The micropropagated plants normally show impaired stomatal mechanism (Capellades, *et al.,* 1990) which results in wilting and death of plantlets (Preece and Sutter, 1991). This results in poor establishment of plantlets during hardening and *ex - vitro* establishment in the field. One of the successes of AMF inoculum production is its ability to promote the growth of host. Hence, the AMF inoculum produced by pot culture method using maize and *A. annua* as the host were tested to determine its efficiency on the growth and establishment of micropropagated ginger under pot culture.

AM fungi play a significant role in ensuring the health of tissue culture plantlets (Gianinazzi and Gianinazzi-Pearson, 1988) by reducing the acclimitisation period of micropropagated plants (Salamanca *et al.,* 1992). The results on effects of different sources of *G. intraradices* inoculum indicated that *A. annua* based inoculum (T2) recorded maximum survival percentage (100), number of leaves (21.0), plant height (28.32 cm), number of tillers (2.4), fresh weight (9.631), and dry weight of plant (0.965). In the present study, the *A. annua* based inoculum performed better than the maize based inoculum of *G. intraradices* under unsterile pot culture studies.

The AM fungi are known to show a preferential colonisation to the host and hence the extent to which the host benefits depend on the fungal species involved in the symbiosis (Abbott and Robson, 1982).

The *A. annua* based *G. intraradices* inoculum enhanced the growth and establishment of micropropagated ginger. These findings are in agreement with earlier studies where it was reported that *Glomus* spp. enhanced the growth and establishment of micropropagated ginger (Surendra-Gopal, 2009). The AMF improved the growth of micropropagated jackfruit (Ramesh, 1990) and banana plantlets (Rizzardi, 1990). It clearly indicates that the efficient AM fungi need to be selected for a particular host with a specific fungal inoculum. Earlier reports have shown that AMF species or strains of AM fungi can vary in their capacity to take up nutrient (P) from soil and transfer it to the host plant (Burleigh *et al.,* 2002, Smith *et al*., 2000). There is high functional diversity among AM fungal species that results in variation in plant responses (Munkrold *et al.,* 2004).

The present studies on *Agrobacterium rhizogenes* mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua*, indicated no *G. intraradices* infection on the *A. rhizogenes* mediated hairy roots of *A. annua* under both solid (static condition) and liquid media (shaking condition). Microbial contamination during AMF spore inoculation was the main limiting factor for proliferation. Thus the procedure for *in vitro* inoculation of AMF spores in hairy roots of *A. annua*, are yet to be standardised. A suitable medium that will allow survival and proliferation of hairy roots and AMF together also needs to be standardised.

Future line of work

- Control of the contaminants during AMF infection on hairy roots under *in vitro*.
- Selection of suitable medium for co - cultivation of AM fungi with hairy root.
- Inoculation protocol for AMF on hairy root under liquid media needs to be refined.

Summary

SUMMARY

The summary of the study on "*Agrobacterium rhizogenes* mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L." carried out at the Centre for Plant Biotechnology and Molecular Biology of the College of Horticulture, Vellanikkara are presented below:

1. *Glomus intraradices* infection was confirmed in *Artemisia*.
2. Percent root colonisation and spore count was confirmed in *Artemisia*.
3. Hairy roots were induced with two strains of *Agrobacterium rhizogenes* and MTCC 532 was superior to MTCC 2364
4. *Glomus intraradices* spores germinated (17.3%) on water agar media supplemented with 0.16 mg l^{-1} cellulase enzyme for inoculation on hairy roots.
5. Normal procedures carried out to infect hairy roots of *Artemisia* with normal and germinated spores of AMF did not yield positive results. The hairy roots failed to proliferate further and the AMF inoculum also did not survive even after repeated attempts in different culture conditions.
6. *A. annua* based soil inoculum recorded maximum survival per cent, number of leaves, plant height, number of tillers, plant fresh and dry weight under pot culture studies with TC derived ginger plants. Eventhough *Glomus intraradices* did not infect hairy roots of *Artemisia annua,* the possibility of using *Artemisia annua* as a host for AMF (*Glomus intraradices*) was confirmed through pot culture experiment.

Conclusion

1. The studies on the effect of *G. intraradices* on *Artemisia* confirmed root colonisation and spores in rhizosphere.
2. MTCC 532 is the best suitable strain for hairy induction of Artemisia for this experiment.

3. T_2 *A. annua* based inoculum was most suitable inoculum for growth and establishment of micropropagated ginger with respect to not only in number of leaves, plant height and number of tillers but also in plant biomass.

References

REFERENCES

Abbot, L. K. and Robson, A. D. 1982. Growth of subterranean clover in relation to formation of endomycorrhizas by introduced and indigenous fungi in a field soil. *New Phytol*. 81: 575-585

*Ackermann, C. 1977. Pflanzen aus *Agrobacterium rhizogenes* Tumoren an *Nicotiana tabacum. Pl. Sci.Lett.* 8: 23-30

Ackramann**,** M., Tabatabaei, S. M. F. and Mirmasoumi, M. 2008. Virulence of different strains of *Agrobacterium rhizogenes* on genetic transformation of four *Hyoscyamus* Species. *American-Eurasian J. Agric. & Environ. Sci.,* 3 (5): 759-763

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

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

Alarcon, A., Ferrera-Cerrato, R., Gonzalez-Chavez, M. C., Villegas-Monter, 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

Alkan, N., Gadkar, V., Coburn, J., Yarden, O. and Kapulnik, Y. 2004. Quantification of the arbuscular mycorrhizal fungus Glomus intraradices in host tissue using real – time polymerase chain reaction. *New Phytol.* 161: 877 – 885

Allan, E.J., Eeswara, J.P., Jarvis, A.P., Mordue, A.J., Morgan, E.D and Stuchbury, T. 2002. Induction of hairy root cultures of *Azadirachta indica* A. Juss and heir production of azadirachtin and other important insect bioactive metabolites. *Pl. Cell.Rep*. 21: 374-379

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

Arellano, J., Vazquez, F., Villegas, T. and Hernandez, G. 1996. Establishment of transformed root cultures of *Perzia cuernavacana. Pl. Cell Rep*. 15: 455-458

Ark, P. A. and Thompson, J. P. 1961. Detection of hairy root pathogen, *Agrobacterium rhizogenes*, by the use of fleshy roots. *Phytopathol*. 51: 69 - 71

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., Cantos, M., Troncoso. and Barea, J. M. 1997. Beneficial effect of arbuscular mycorrhizas on acclimatisation of micropropagated cassava plantlets. *Scientia Horticulturae*. 72: 63 - 71

Bapat, V. A. and Ganapathy, T. R. 2005. Hairy roots - a novel source for plant products and improvement. *Natl. Acad. Sci. lett.* 28: 61-68

Batra, J., Dutta, A., Singh, D., Kumar,S. and Sen, J. 2004. Growth and terpenoid production in *Catharanthus roseus* hairy root clones in relation to left and right termini linked Ri T-DNA gene integration. *Pl. Cell. Rep*. 23: 148-154

Becard, G. and Fortin, J. A. 1988. Early events of vesicular - arbuscular mycorrhiza formation on Ri T - DNA transformed roots. *New Phytol.* 108: 211-218

Becard, G. amd Piche, Y. 1990. Physiological features determining vesicular-arbuscular mycorrhizal formation in host and non-host Ri T-DNA transformed roots. *Can. J. Bot*. 68: 1260 – 1264

Benjamin, B.D., Roja, G. and Heble, M.R. 1993. *Agrobacterium rhizogenes* mediated transformation of *Rauwolfia serpentina*: Regeneration and alkaloid synthesis. *Pl. Cell Tissue Organ Cult.* 35: 253-257

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

Bethlenfalvay, G. J. and Newton, W. E. 1991. Agro - ecological aspects of the mycorrhizal, nitrogen fixing legume symbiosis. *The Rhizosphere and plant growth*. Keister, D. L. and Cregan, P. B. (eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands. pp 349 - 354

Bi Yin Li, Wang Hong Gang and Li Xi Ao Lin. 1999. The morphological characteristics of AM symbiosis between arbuscular mycorrhizal fungus and transformed Ri T-DNA carrot roots. *Mycosystema*. 18: 159 - 163

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

Blal, B., Morel, C., Gianinazzi-Pearson, V. S. 1988. Influence of vesicular arbuscular mycorrhizae on phosphate fertilizer efficiency in two tropical acid soils planted with micropropagated oil palm (*Elaeis guineensis* Jacq.) *Biol. Fertil. Soils*. 9; 43-48

Boby, V. U., Balakrishna, A. N. and Bagyaraj, D. J. 2007. Changes in enzyme activity and microbial biomass in the root zone soil of cowpea as influenced by inoculation with AM fungus and soil yeasts. *J. Soil Biol*. 27 (1&2): 118 - 129

Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C. and Barker, D.G. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago*

truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol. Plant-bicrobe Interact.* 6:695-700

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

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

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

Bousquet, J., Simon, L. and Lalonde, M. 1990. DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. *Can. J. For. Res*. 20: 254 – 257

Bresson, W. 2001. The interactive effect of phosphorus and nitrogen on "*in vitro*" spore germination of *glomus etunicatum* becker & gerdemann, root growth and mycorrhizal colonization. *Brazilian J. of Microbiol*. 32: 276-280

Burleigh, S. H., Cavangaro, T. and Jakobsen, I. 2002. Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. *J. Exp. Bot*. 53: 1593 - 1601

Bush, A.L. and Pueppke, S.G. 1991. Cultivar strain specificity between *Chrysanthemum morifolium* and *Agrobacterium tumefaciens. Physiol. Mol. Pl. Pathol.* 39: 309-323

Cai, G.Q., Li, G.Z., Ye, H.C. and Li, G.F. 1995. Hairy root culture of *Artemesia annua* L. by Ri plasmid transformation and biosynthesis of artemesinin. *Chinese J. Biotech*. 1: 315-320

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

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

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

Celma, C.R., Palazim, J., Cusido, R.M. Pinol, M.T. and Keil, M. 2001. Decreased scopolamine yield in field grown *Dubosia* plants regenerated from hairy roots. *Planta Med*. 67:249-253

Chabaud, M., Becard, G. and Barker, D. G. 2006. Targeted inoculation of *in vitro* Ri T-DNA propagated roots with pregerminated *Gigaspora* spores, *Medicago truncatula* Handbook. pp 3 - 4

Chabot, S., Becard, G. and Piche, Y. 1992. Life cycle of *Glomus intraradices* in root organ culture. *Mycologia*. 84: 315 - 321

Chandran, R. P. and Potty, V. P. 2008. Induction of hairy roots through the mediation of four strains of *Agrobacterium rhizogenes* on five host plants. *Indian J. Biotech.,* 7: 122 – 128

Chaudhuri, K.N., Ghosh, B., tepfer, D. and Jha, S. 2005. Genetic transformation of *Tylophora indica* with *Agrobacterium rhizogenes* A4: growth and tylophorine productivity in different transformed root clones. *Pl. Cell. Rep*. 24: 25-35

Chen, D.H., Ye, H.C., Li, G.F and Liu, B.Y. 1999. Expression of green fluorescent protein gene in transgenic shoots of *Artemesia annua. Acta. Bot. Sin.* 19: 1097-1106

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 clover growing in calcareous soil spiked with various quantities of zinc. *Chemosphere*. 50: 839 - 846

Chilton, M., Tepfer, D., 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

Christen, P., Aoki, T. and Shimomura, K. 1992. Characteristics of growth and tropane alkaloid production in *Hyoscyamus albus* hairy roots transformed with *Agrobacterium rhizogenes* A_4. *Pl. Cell Rep*. 11: 597 - 600

Christey, M.C. 2001. Use of Ri-mediated transformation for production of transgenic plants. *In Vitro cell Dev. Biol. Pl*. 37: 687-700

Clark, R. B. and Zeto, S. K. 1996. Growth and root colonisation of mycorrhizal maize grown on acid and alkaline soil. *Soil Biol. Biochem*. 28: 1501 - 1511

Comprubi, A., Estuan, V., Calvet, C. and Pera, J. 1990. Infectivity and effectivity of *Glomus mosseae* in four different species of medicinal plants. *Symbiosis*. 9: 305 - 307

Dalpe, Y. and Declerck, S. 2002. Development of *Acaulopora rehmii* spore and hyphal swellings under root-organ culture. *Mycologia*. 94: 850 - 855

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

Davies, F. T. Jr., Portugal-Olalde, V., Aguilera-Gomez, L., Alvarado, M. J., Ferrera-Cerrato, R. C. and Bouton, T. W. 2002. Alleviation of drought stress of Chile ancho pepper (*Capsicum annuum* cv San Louis) with arbuscular mycorrhiza indigenous to Mexico. *Scientia Hortuculturae*. 92: 347 - 359

Dayala, D., Bagyaraj, D. J. and Aparna, J. 2007. Germination of arbuscular mycorrhiza fungal spores using cellulase enzyme and cell free extract of bacteria and fungi. *J. Soil Biol*. 27 (1&2):111 - 117

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

Declerck, S. and Angelo Van Coppenholle, M. G. 2000. Cryopreservation of entrapped monoxenically produced spores of an arbuscular mycorrhizal fungus. *New Phytol*. 148: 176-196

Dell'Amico, J., Torrecillas, A., Rodruguez, 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

Dernier, A. B., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C. and Barker, D. G. 2001. *Agrobacterium rhizogenes*- transformed Roots of *Medicago truncatula* for the Study of Nitrogen-Fixing and Endomycorrhizal Symbiotic Associations. MPMI. 14 (6): 695 – 700

Devey, M.R., Mulligan, B.J., Gaitland, K.M.A., Peel, E., Saegent, A.W. and Morgan, A.J. 1987. Transformation of *Solanum* and *Nicotiana* species using an Ri plasmid vector. J. Exp. Bot. 38: 1507-1516

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

Dobigny, A., Ambroise, A., Haicour, R., David, C., Rossignol, L. and Sihachakr, D. 1995. Transformation of potato using mannopine and cucmopine strains of *Agrobacterium rhizogenes*. *Pl. Cell Tissue Organ Cult*. 40: 225-230

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

Doss, D. D., Bagyaraj, D. J. and Aparna, J. 2008. Co-cultivation of arbuscular mycorrhizal fungi with *Agrobacterium rhizogenes* mediated Ri T-DNA transformed hairy roots of tomato explants. *J. Soil Biol. Ecol*. 28 (1&2): 70-76

Douds, D. and Becard, G. 1993. Competitive interaction between *Gigaspora margarita* and *Gigaspora gigantia in vitro*. In: Proceedings of the Ninth North American Symposium on Mycorrhizae, Guelph, ON, Canada, August 8-12

Durang-Tardif, M., Broblie, R., Slightom, J. and Tepfer, D. 1985. Structure and expression of TR-DNA from *Agrobacterium rhizogenes* and *Nicotiana tabacum* organ and phenotypic specificity. *J. Mol. Biol*. 186: 557-564

Edwards, U., Rogall, T., Blöcker, H., Emde, M. and Böttger, E. C. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterisation of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* 17: 7843 – 7853

Elmeskaoui, A., Damont, J. P., Poulin, M. J., Piche, Y. and Desjardins, Y. 1995. A tripartite culture system for endomycorrhizal inoculation of micropropagated strawberry plantlets *in vitro*. *Mycorrhiza*. 5: 313-319

Eranna, N., Bagyaraj, D. J. and Gowda, B. 1998. VA mycorrhizal association in medicinal plants of south eastern dry zone of Karnataka. *J. Soil Biol. Ecol*. 18 (1&2): 30-36

Estrada - Luna, A. A. and Davies, F. T. 2003. Arbuscular mycorrhizal fungi influence water relations, gas exchange, abscisic acid and growth of micropropagated

Chile ancho pepper (*Capsicum annum*) plantlets during acclimatisation and post acclimatisation. *J. Pl. Phisol*. 160: 1073 - 1083

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

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? *Cultivor Tropicales*. 23 (3): 47 - 52

Flores, H.E., Dai, Y., Cuello, J.L., Ignacio, E., Mendoza, M and Loyola-Vargas, K. 1993. Green roots: photosynthesis and photoautotrophy in an underground plant organ. *Pl. Physiol.* 11: 363-371

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

Fortin, J. A., Becard, G., Declerck, S., Dalpe, Y., St. Arnaud, M., Coughlan, A. P. and Piche, Y. 2002. Arbuscular mycorrhiza in root organ cultures. *Can. J. Bot*. 80: 1 - 20

Fortunato, I. M. and Avato, P. 2008. Plant development and synthesis of essential oils in micropropagated and mycorrhizal inoculated plants of *Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart. *Plant Cell Tiss. Organ. Cult*. 93: 139 – 149

Gamborg, O. L. and Shyluk, J. P. 1981. Nutrition, media and characteristics of plant cell and tissue cultures. *Plant Tissue Cultures: Methods and Application in Agriculture* (ed. Thorpe, T. A). Academic Press, New York, p. 21 - 24

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

Gerdemann, G. W. and Nicolson, T. H. 1963. Spores of mycorrhizal endogene species extracted from soil by wet-sieving and decanting. *Trans. Br. Mycol. Soc*, 46: 235 - 244

Gianinazzi, S. and Gianinazzi-Pearson, V. 1988. Mycorrhizae; a plant's health insurance. Chimica Oggi, Octobre, France. 56-58

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

Giri, A., Ravindra, S.T., Dhingra, V. and Narasu, M.L. 2001. Influence of different strain of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemesia annua. Current Sci*. 81: 378-382

Godwin, I., Todd, G., Ford-Lloyd, B. and John-Newbury, H. 1991. The effect of acetosyringone and pH in *Agrobacterium* mediated transformation vary according to plants species. *Pl. Cell Rep*. 9: 671-673

Gonzales**,** D.L. and Weathers, P.J. 2003. Tetraploid *Artemesia annua* hairy roots produced more artemisinin than diploids. *Pl. Cell Rep*. 21: 809-813

Gonzalez-Chavez, C., D'Haen, J., Vangronsveld, J. and Dodd, J. C. 2002. Copper sorption and accumulation by the extra redical mycelium of different *Glomus* spp. 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 micropropagated sugarcane. *Ann. Agric. Bio. Res*. 6 (1): 19 - 22

Granger, R. L., Plenchette, 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

Gribaudo, I., Schubert, A. and Camino, C. 1995. Establishment of grapevine axenic root lines by inoculation with *Agrobacterium rhizogenes*. *Adv. Hort. Sci*. 9: 87-91

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

Grout, B. 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 mycorrhizal to biological protection of micropropagated pineapple (*Ananas comosus* L. Merr.) against *Phytophthora cinnamomi* R and S. *Agric. Sci*. Finland. 3: 241 - 251

Guo, Y., George, F. and Marschner, H. 1996. Contribution of an arbuscular mycorrhizal fungus to uptake of Cadmium and Nickel in bean by maize plants. *Pl. Soil*. 184: 195 - 205

Guttay, 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

Hamby, R. K., Sims, L., Issel, L. and Zimmer, E. 1988. Direct ribosomal RNA sequencing: optimisation of extraction and sequencing methods for work with higher plants. *Plant Mol. Biol. Reporter*. 6: 175 - 192

Hanischten-Cate, C.H., Loonen, A.E., Ottavani, M.P., Enniklvn-Eldik, G. and Stiekema, W.J. 1990. Frequent spontaneous deletions of Ri-T-DNA in

Agrobacterium rhizogenes transformed potato roots and regenerated plants. *Pl. Mol. Biol.* 14: 735-741

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, L. L. and Smith, S. E. 1983. *Mycorrhizal Symbiosis*. Academic Press, London, 288 p

Hawes, M.C., Smith, L.Y. and Howarth, A.J. 1998. *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Mol. Pl. Microb. Interaction*. 1: 182-190

Henson, J. M., Goins, T., Grey, W., Mathre. and Elliott, M. L. 1993. Use of polymerase chain reaction to detect *Gaumannomyces graminis* DNA in plants grown in artificially and naturally infested soil. *Phytopathology*. 83: 283 – 287

Hepper, C. M. and Smith, G. A. 1976. Observation on the germination of *Endogene* spores. *Trans. Br. Mycol. Soc*. 66: 189-194

Hepper, C. M. 1978. Techniques for studying the infection of plants by vesicular-arbusczular mycorrhizal fungi under axenic conditions. *New Phytol*. 93: 537-542

Hilderbrand, E.M. 1934. Life history of hairy root organism in relation to its pathogenesis on nursery apple trees. *J. Agric. Sci*. 48: 857-885

Hilton, M.G. and Wilson, P.D.G. 1995. Growth and uptake of sucrose and mineral ions by transformed root cultures of *Datura stramomium, Datura candida, Datura wrightii, Hyoscyamus muticus and Atropa belladonna. Planta Med*. 61: 345-350

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

Hu, Z.B. and Alfermann, A.W. 1993. Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza. Phytochemistry*. 32: 699-703

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

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 until. *Int. J. Phytoremed*. 4 (3): 203 - 221

Jarsfer, A. G. and Sylvia, D. M. 1994. Aeroponic culture of VAM fungi. Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology. Varma, A. K. and Hock, B. (eds.). Springer-Verlag, Berlin. pp 427 - 441

Jaziri, M., Yoshimatsu, K., Homes, J. and Shimomura, K. 1994. Traits of transgenic *Atropa belladonna* doubly transformed with different *Agrobacterium rhizogenes* strains, *Plant Cell Tissue Organ Cult*. 38: 257 - 263

Jaziri, M., Shimomura, K., Yoshimatsu, K. and Fauconnier, M. L. 1995. Establishment of normal and transformed root cultures of *Artemisia annua* L. for artemisinin production. *J. Pl. Physiol*. 14: 175-177

Jeffries, P., Gianinazzzi, 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 soils*. 37: 1 – 16

John, N. K. and Miranda, M. H. 2002.Colonisation of roots by arbuscular mycorrhizal fungi using different sources of inoculum.*Mycorrhiza*.12:181-184

Joseph, P. J. 1997. Management of rhizome rot of ginger using VA mycorrhizae and antagonists. Ph.D. thesis, Kerala Agricultural University, Thrissur. 192 p

Jung, G. and Tepfer, D. 1987. Use of genetic transformation by the Ri T-DNA of *Agrobacterium rhizogenes* to stimulate biomass and tropane alkaloid production in *Atropa belladonna* and *Calystegia sepium* roots. *Pl. Sci*. 50: 145-151

Karandashov, V., Kuzvkina, I., Hawkins, H. J. and George, E. 1999. Growth and sporulation of the mycorrhizal fungus *Glomus caladonium* in dual culture with transformed carrot roots. *Mycorrhiza*. 10: 23-28

Karmarkar, S. H. 2001. Genetic transformation and hairy root culture in ada-kodien (*Holostemma ada-kodien* K. Schum). M. Sc. (Horticulture) thesis, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, 137 p

Karmarkar, S. H. and Keshavachandran, R. 2005. Hairy root induction in *Holostemma ada-kodien* K. Schum with *A. rhizogenes*. Proceedings of ICAR National Symposium on Biotechnological interventions for improvement of Horticultural crops: Issues and strategies; 10-12 Jan. 2005; Thrissur, Kerala. pp. 350-351

Karthikeyan, A., Palanivel, S. Parvathy, S. and Bhakyaraj, R. 2007. Hairy root induction from hypocotyl segments of groundnut (*Arachis hypogaea* L.). *African J. of Biotechnol.,* 6 (15): 1817-1820

Kaya, C., Higgs, D., Kirnak, H. and Tas, I. 2003. Mycorrhizal colonisation improves fruit yield and water use efficiency in water melon (*Citrullus lanatus* Thunb) grown under well-watered and water-stressed conditions. *Pl. Soil*. 253: 287 - 292

Khaliq, A. and Bagyaraj, D. J. 2000. Colonisation of AMF on Ri - T - DNA transformed roots in synthetic medium. *Indian J. Exp. Biol*. 38: 1147 – 1151

Khaliq, A., Gupta, M. L. and Kumar, S. 2001. The effect of VAM fungi on growth of peppermint. *Indian Phytopath*. 54: 82 - 84

Kiernan, J. M., Hendrix, J. W., Stoltz, L. P. and Maronek, D. M. 1984. Characterisation of strawberry plants produced by tissue culture and infected with specific mycorrhizal fungi. *Hort. Sci*. 19: 883 - 885

Kintzios, S. E. 2002. The biotechnology of oregano (*Origanum* sp. and *Lippia* sp.). In: Kintzios, S. E. (ed) Medicinal and aromatic plants - industrial profiles, Oregano. The genera *Origanum* and *Lippia*. Vol.25. Taylor and Francis, London. pp. 237 - 242

Lata, H. De Andrade, Z., Schaneberg, B., Bedir, E., Khan, L. and Moraes, R. 2003. Arbuscular mycorrhizal inoculation enhances survival rates and growth of micropropagated plantlets of *Echinacea pallida*. *Planta Vedica*. 69: 679 - 682

Lee, N., Wetstein, H. Y. and Sommer, H. E. 1985. Effect of quantum flux density on photosynthesis and chloroplast ultra structure in tissue culture plantlets. *Pl. Physiol*. 78: 637 - 641

Lee, M. H., Yoon, E. S., Jeong, J. H. and Choi, Y. E. 2004. *Agrobacterium rhizogenes* mediated transformation of *Taraxacum platycarpum* and changes of morphological characters. *Pl. Cell Rep*. 22: 822-827

Lesham, 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

Leu, S. W., Lian, S. T. and Chang, D. C. N. 1994. *In vitro* sporulation of vesicular-arbuscular mycorrhizal fungi, *Gigaspora gigantica* and *Glomus mossea*e by dual culture with carrot Ri T-DNA transformed root and tomato root culture. *Mem. Coll. Agric*. 1 - 8 November, 1994. National Taiwan University, Taiwan, 34: 21 - 32

Li Min, Li Pengqi. And Liu Runjin. 2002. Effect of AMF on micropropagated taro (*Colocasia esculenta*). *Acta Horticulturae Sinica*. 29: 451 – 453

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, W., Asada, Y. and Yoshikawa, T. 2000. Flavanoid constituents from *Glycyrrhiza glabra* hairy root cultures. *Phytochemistry*. 55: 447-456

Li, Z. T., Dhekney., Dutt, M. and Gray, D. J. 2008. An improved protocol for Agrobacterium – mediated transformation of grapevine (*Vitis vinifera* L.). *Plant Cell Tiss. Organ. Cult*. 93: 311 - 321

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: 847 - 853

Limani, M. A., Sun, L. Y., Douat, C., Helgeson, J. and Tepfer, D. 1998. Natural genetic transformation by *Agrobacterium rhizogenes*. *Pl. Physiol*.118:543-550

Lins, G. M., De, L., Trindade, A. V. and Rocha, H. S. 2003. Utilisation of *Gigaspora margarita* in micropropagated banana plants in different stages of rooting. *Revista Brasileira de Fruticultura*. 25 (1): 143 - 147

Liu, B., Ye, H., Li, G., Chen, B., Geng, S., Zhang, Y., Chen J. and Gao, J. 1998. Studies on dynamics of growth and biosynthesis of artemisinin in hairy roots of *Artemisia annua* L. *Chinese J. Biotech*. 14: 249 - 254

Liu, A., Hamel, C., Elmi, A., Costa, C., Ma, B. and Smith, D. L. 2002. Concentrations of K, Ca, and Mg in maize colonised by arbuscular mycorrhizal fungi under field conditions. *Can. J. Soil Sci*. 82: 271 - 275

Lopez, A., Pinchet, J., Fernandez, C., Calvert, C. and Comprubi, A. 1997. Growth response of OHF - 333 pear rootstock to arbuscular mycorrhizal fungi,

phosphorus nutrition and *Pratylenchus vulnus* infection. *Fundament. Appl. Nematol*. 20: 87 - 93

Lorence, A., Bolivar, M. F. and Nessler, C. L. 2004. Camptothecin and 10-hydroxy camptothecin from *Camptotheca acuminate* hairy roots. *Pl. Cell Rep*. 22: 437-441

Lovato, P., Guillemin, J. P. and Gianinazzi, S. 1994. Application of commercial arbuscular endomycorrhizal fungal inoculants to the establishment of micropropagated grapevine rootstock and pineapple plants. *Agronomie*. 12: 673 - 680

Lubraco, G., Schubert, A. and Prevaiti, A. 2000. Micpropagation and mycorrhization of *Allium sativum*. *Acta Horticulturae*. 530: 339 - 343

Malarz, J., Stojakowska, A. and Kisiel, W. 2002. Sesquiterpene lactones in hairy root cultures of *Cichorium intybus*. *Naturforsch*. 57: 994-997

Mano, Y., Ohkawa, H. and Yamda, Y. 1986. Production of tropane alkaloids by hairy root cultures of *Dubosia leichardtii* transformed by *Agrobacterium rhizogenes*. *Pl. Sci*. 59: 191-201

Marx, D. H. 1975. Mycorrhizae and the establishment of trees in strip-mined land. *Ohio J. Sci*. 75: 88 - 97

Mathur, N. and Vyas, A. 1999. Improved biomass production, nutrient uptake and establishment of *in vitro* raised *Zizyphus mauritiana* by VA mycorrhiza. *J. Pl. Physiol*. 155: 129 - 132

Matylda Sidwa-Gorycka, Krolicka, A., Orlita, A., Malinski, E., Golebiowski, M., Kumirska, J., Chromik, A., Biskup., E., Stepnowski, P. and Lojkowska, E. 2009. Genetic transformation of *Ruta graveolens* L. by *Agrobacterium*

rhizogenes: hairy root cultures a promising approach for production of coumarins and furanocoumarins. *Plant Cell Tiss. Organ Cult*. 97:59 - 69

Miller, R. M. and Jastrow, J. D. 1990. Hierarchy of root and mycorrhizal fungal interaction with soil aggregation. *Soil Biol. Biochem*. 22: 579 – 584

Momocilovic, I., Grubisic, D., Kojic, M. and Neskovic, M. 1997. *Agrobacterium rhizogenes* mediated transformation and plant regeneration of four *Gentiana* species. *Pl. Cell Tissue and Organ Cult*. 50: 1-6

Moore, L., Warren, G. and Strobel, G. 1974. Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. *Plasmid*. 2: 617-626

Morton, J. B. and Benny, G. L. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order Glomales, two new suborders, Glominae and Gigasporinae 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 contions. *Gen. Microbiol*. 27: 509-520

Mosse, B. 1973. Advances in the study of vesicular arbuscular mycorrhiza. *Rev. Phytopath*. 11: 171 - 196

Mosse, B. and Hepper, C. M. 1975. Vesicular arbuscular infection infection in root organ cultures. *Physiol. Pl. Path*. 5: 215 – 223

Mosse, B. and Thompson, J. P. 1984. Vesicular-arbuscular endo mycorrhizal inoculum production: Explanatory experiments with beans (*Phaseolus vulgaris*) in nutrient flow culture. *Can. J. Bot*. 62: 1523 – 1530

Mugnier, J. and Mosse, B. 1987.Vesicular-arbuscular mycorrhizal infections in transformed Ri T-DNA roots grown axenically. *Phytopathology*.77: 1045 - 1050

Mukherjee, S., Ghosh, S. B., Jha, T. B. and Jha, S. 1995. Genetic transformation of *Artemisia annua* by *Agrobacterium rhizogenes*. *Indian J. Exp. Biol*. 33: 868 - 871

Munkrold, L., Kjoller, R., Vestberg, M., Rosendahl, S. and Jakobsen, I. 2004. High functional diversity within species of arbuscular mycorrhizal fungi. *New Phytol*. 164: 357 - 364

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Pl*. 15: 473 - 497

Naverrete, G. E., Affantranger, X. A., Olivares, J. E., Camino, C. D., Santana, O., Murillo, E., Guillén, G., Guevara, N. S., Acosta, J., Quinto, C., Li, D., Gresshoff, P. M. and Sánchez, F. 2006. *Agrobacterium rhizogenes* Transformation of the *Phaseolus* spp.: A Tool for Functional Genomics. MPMI. 19 (12): 1385 - 1393

Nilsson, O. and Olsson, O. 1997. Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. *Physiol. Pl*. 100: 463-473

Noda, T., Tanaka, N., Mano, Y., Nabeshima, S., Ohkawa, H. and Matsui, C. 1987. Regeneration of horse radish hairy roots incited by *Agrobacterium rhizogenes*. *Pl. Cell Rep*. 6: 283-286

Oksman-Caldentey, K. M., Sevon, N., Vanhala, L. and Hiltunen, R. 1994. Effect of nitrogen and sucrose on the primary and secondary metabolism of transformed root cultures of *Hyscyamus muticus*. *Pl. Cell Tissue and Organ Cult*. 38: 263-272

Ooms, G., Karp, A., Burell, M. M., Twell, D. and Roberts, J. 1985. Genetic modification of potato development using Ri T-DNA. *Theor. Appl. Genet*. 70: 440 - 446

Ottaviani, M. P., Schel, J. H. N. and Hanischten-Cate. 1990. Variation in structure and plant regeneration of *Agrobacterium rhizogenes* transformed and control roots of potato. *Pl. Cell Tissue and Organ Cult*. 20: 25-34

Panse, V. G. and Sukhatme, P. V. 1978. *Statistical methods for agricultural workers*. Indian Council of Agricultural Reasearch, New Delhi. p. 347

Patil, N. N. 2007. Influence of AM fungi on growth and nutrition of egg plant. *J. Soil Biol*. 27 (1&2): 106 - 110

Pawar, P. K. and Maheshwari, V. L. 2004. *Agrobacterium rhizogenes* mediated hairy root induction in two medicinally important members of family Solanceae. *Indian J. Biotech*. 3: 414-417

Pawlowska, E. T., Doudsjrand, D. D. and Charwat, I. 1999. *In vitro* propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. *Mycol. Res*. 103: 1549-1556

Payne, G. F., Bringi, V., Prince, C. and Shuler, M. l. 1992. Root cultures. Shuler, M. L. (ed). *Hanser series in biotechnology*. *Plant cell and tissue culture in liquid systems*. Hanser, Munich, pp 225-279

Pfleger, E. L., Stewart, E. L. and Noyd, R. K. 1994. Role of VAM fungi in mine land revegetation. In: *Mycorrhizae and Plant Health*. Pfleger, E. L. and Linderman, P. G. (eds.). APS, Minnesota, USA, pp 47 - 81

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. Mycol. Soc*. 55: 158 – 161

Pierick, R. L. M. 1987. *In Vitro Culture of Higher Plants*. Martinus Nijhoft Publishers, Boston, 344 p.

Plenchette, C., declereck, S., Diop, T. A. and Strullu, D. G. 1996. Infectivity of monoxenic subcultures f the AM fungs *Glomus versiforme* associated with Ri T-DNA transformed carrot roots. *Appl. Microbiol. Biotechnol*. 46: 545-548

Potty, V. P. 2001. Growth and establishment of *Glomus microcarpum var. microcarpum* in genetically transformed roots of cassava. Proc. VCBN Network Meeting, November 4-9, 2001. Taylor, N. J., Ogbe, F. and Eanguet, C. M. (eds.), St. Luis, USA, pp 5 - 18

Potty, V. P. and Pratapchandran, R. 2004. Production of transgenic hairy roots for culturing mycorrhizal fungi. *Proc. Sixteenth Kerala Sci. Cong*. January 29 - 31, 2003 (ed. Potty, V. P.), Kozhikode, pp. 380 - 385

Preece, J. E. and Sutter, E. G. 1991. Acclimatisation of micropropagated plants to the greenhouse and field. *Micropropagation: Technology and Applications* (eds. Debergh, P. C. and Zimmerman, R. H.). Academic Publishers, Dordrecht, The Netherland, pp 71 - 93

Rahimi, K., Haghbeen, K., Marefatjo, J., Jazii, F. R. and Sheikhani, R. 2008. Successful production of hairy roots of *Valeriana sisymbriifolium* by *Agrobacterium rhizogenes*. *Biotechnol.,* 7 (2): 200 - 204

Rai, M. K. 2001. Current advances in mycorrhisation in micropropagation. *In vitro Cell Dev. Biol*. 37: 158-167

Raman, N. and Sambandan, K. 2000. Axenic germination of *Scutellospora erithropa* and *Scutellospora nigra* in *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

Ramesh, B. 1990. *Ex vitro* establishment of jack plantlets (*Artocarpus caryophyllus*). M. Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur. 52 p

Rancillac, M. Cadoux, F., Leduc, D. and Kahane, R. 1996. Improvement of a protocol to establish *in vitro* arbuscular mycorrhizal strains with *in vitro* bulbs of onion, *Allium cepa* L. In: Novel biotechnological approaches to plant production: from sterile root to mycorrhizosphere. Joint COST meeting 8.21, Pisa, Itali. 32

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 fungi in Indian arid zone. *J. Arid Environ*. 51 (1): 113 - 123

Rathore, T. S., Annapurna, D. and Joshi, G. 2007. Effect of vesicular arbuscular mycorrhzae (VAM), VAM helper bacteria and nitrogen fixing bacteria on seedling growth of *Santalum album* L. in root trainers. *J. Soil Biol*. 27 (1&2): 96 - 105

*Rech, E. L., Golds, T. J., Husnain, T., Vainstein, M. H., Jones, B., Hammatt, N., Mulligan, B. J. and Davey, M. R. 1989. Expression of a chimaeric resistance gene introduced into the wild soybean *Glycine canescens* using a cointegrated Ri plasmid vector. *Plant Cell Rep*. 8: 3 - 36

Reuther, S. 1986. Characteristics of plants produced in tissue culture and their effects on further cultures. *Zier Pflan*. 26: 172 - 174

Rhodes, L. H. 1980. The use of mycorrhizae in crop production system. *Outl. Agric*. 10: 275 – 281

Rhodes, M. J.C., Parr, A.J., Giuletti, A. and Aird, E. L.H. 1994. Influence of exogenous hormones on the growth and secondary metabolite formation in transformed root cultures. *Plant Cell Tiss. Org. Cult*. 38:143-151

*Ricker, A. J., Banfield, W. M., Wright, W. H., Keit, G. W. and Sage, H. E. 1930. Studies on infectious hairy roots of nursery apple trees. *J. Agric. Res*. 41: 507 - 540

Rillig, M. C. and Steinberg, P. D. 2002. Glomalin production by an arbuscular mycorrhizal fungus: a mechanism of habitat modification. *Soil Biol. Biochem*. 34: 1371 - 1374

Rizzardi, V. 1990. Effect of inoculation with VA mycorrhizal fungi on the growth of micropropagated *Musa acuminate* clone Grand Naine. *Revista de Agricultura Subtropicale e tropicale*. 84: 483 - 848

Rufykiri, G., Thiry, Y., Wang, L., Delvaux, B. and Declerck, S. 2002. Uranium uptake and translocation by the arbuscular fungus, *Glomus intraradices*, under root-organ culture conditions. *New Phytol*. 156: 275 - 281

Ruiz-Lozano, J. M., Roussel, H., Gianinazzi, S. and Gianinazzi-Pearson, V. 1999. Defense genes are differentially induced by a mycorrhizal fungus and *Rhizobium* sp. in a wild-type and symbiosis-defective pea genotypes. *Mol. Pl. Microbe Interact*. 12: 976 - 984

Safir, G. R., Boyer, J. S. and Gerdemann, J. W. 1972. Nutrient status and mycorrhizal enhancement of water transport in soybean. *Pl. Physiol*. 19: 700 - 703

Salamanca, C. P., Herrea, M. A. and Barea, J. M. 1992. Mycorrhizal inoculation of micropropagated woody legumes used in revegetation programmes for desertified Mediterranean ecosystems. *Agronomie*. 12: 869-872

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor, New York.

Sanchez-Blanco, M. J., Ferrandez, T., Morales, M. A., Morte, A. and Alarcon, J. J. 2004. Variations in water status, gas exchange and growth in *Rosmarinus officinalis* plants infected with *Glomus desrticola* under drought conditions. *J. Pl. Physiol*. 161: 675 - 682

Sauerwein, M., Wink, M. and Shimomura, K. 1992. Influence of light and phytohormones on alkaloid production in transformed root cultures of *Hyoscymus albus*. *J. Pl. Physiol*. 140: 147-152

Schellenbaum, L., Berta, G., Ravolanirisa, F., Tisserant, B., Gianinazzi, S. and Fitter, A. H. 1991. Influence of endomycorrhizal infection on root morphology in a micropropagated woody plant species *Vitis vinifera* (L.). *Ann. Bot*. 68: 135 – 234

Schenk, N. C. 1982. *Methods and Principles of Mycorrhizal Research.* American Phytopathological Society, St. Paul, Minnesota, USA, pp 47 - 54

Schubert, A., Mazzitelli, M. and Gribaudo, I. 1987. Effect of inoculation with vesicular arbuscular mycorrhizal fungi on micropropagated *Vitis berlandieri* and *Vitis rifaria* Kober 5 BB. Proc. Symp. Florizel, Arlon, Belgium. 87: 144-153

Severn - Eillis**,** A. 1999. Microorganisms may improve growth of micropropagated banana plants. *Neltropika Bull*. 303: 35 - 36

Sevon, N. and Oksman-Caldentey, K. 2002. *Agrobacterium rhizogenes* - mediated transformation: Root cultures as a source of alkaloids. *Planta Med*. 68: 859-868

Shaneeja, V. M. 2007. Genetic transformation in *Artemisia annua* L. for hairy root induction and enhancement of secondary metabolites. M. Sc. Thesis. Kerala Agricultural University, Thrissur, Kerala, India. 164 p

Shi, H. P. and Kintzios, Y. P. 2003. Genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes* and puerarin production in hairy roots. *Pl. Cell Rep*. 21: 1103-107

Simon, L., Lalonde, M. and Bruns, T. D. 1992a. Specific amplification of 18S fungal ribosomal genes from vesicular - arbuscular endomycorrhizal fungi colonising roots. *Appl. Environ. Microbial.* 58: 291 - 295

Simon, L., Lévesque, M. and Lalonde, M. 1992b. Rapid quantitation by PCR of endomycorrhizal fungi colonising roots. *PCR Methods. Applic*. 2: 76 - 80

Simon, L., Lévesque, M. and Lalonde, M. 1993. Identification of endomycorrhizal fungi colonising roots by fluorescent single - strand conformation polymorphism – polymerase chain reaction. *Appl. Environ. Microbial.* 59: 4211 - 4215

Sinker, V., Pythoud, F., White, F., Neste, E. and Gordon, M. 1988. *Rol* A locus of the Ri plasmid directs developmental abnormalities in transgenic plants. *Genes Dev*. 2: 688-698

Sivaprasad, P., Ramesh, B., Mohanakumaran, N., Rajmohan, K. and Joseph, P. J. 1995a. VA mycorrhiza for the *ex vitro* establishment of tissue culture plantlets. *Mycorrhiza: Biofertlizer for the future*. (eds. Adholeya A. and Singh, S.). *Proc. Third Nat. Conf. 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 - climatic conditions. March 5 - 7, 1999, Bhopal, India.

Sivaprasad, P. and Sulochana, K. K. 2004. Integration of arbuscular mycorrhizal technology with micropropagation. FIB, Govt. of Kerala, Thiruvananthapuram, 22 p

Smith, G. S., Johnston, C. M. and Cornforth, I. S. 1983. Comparision of nutrient solutions for growth of plants in sand culture. *New Phytol*. 94: 537 - 548

Smith, S. E. and Read, D. J. 1997. Mycorrhizal Symbiosis. Academic press, San Deigo, California, 342 p

Smith, S. E., Smith, F. A. and Jakobsen, I. 2000. Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology*, 133: 16 - 20

Sogin, M. L., Gunderson, J. H., Elwood, H. J., Alonso, R. A. and Peattie, D. A. 1989. Phylogenetic meaning of the kingdom concept: an unusual ribosomal RNA from *Giardia lamblia*. *Science*. 243: 75 - 77

Sreelatha, V. 1992. Improvement of propagation efficiency of *Anthurium* spp. *in vitro*. Ph. D. thesis, Kerala Agricultural University, Thrissur, 119 p

Stachel, S.E., Messens, E., Van Montagu, M. and Zambryski, P**.** 1985. ldentification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature.* 318**:** 624-629

Steinberg, P. D. and Rillig, M. C. 2003. Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. *Soil Biol. Biochem*. 35: 191 - 194

Stiller, J., Martirani, L., Tuppale, S., Chian, R. J., Chiurazzi, M., and Gresshoff, P. M. 1997. High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*. *J. Exp. Bot.* 48:1357-1365

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

Surendra-Gopal, K. 2009. Development of plant growth promoting microorganisms consortia technology for *ex vitro* establishment of micropropagated vanilla (*Vanilla planifolia*. Andr.) and ginger (*Gingiberra officinale*. Rosc.). Final report submitted to Kerala State Council for Science Technology and Environment. Government of Kerala. 122 p

Surendra-Gopal, K., Sally K Mathew. and Nazeem, P. A. 2009. *Glomus* Spp. - A suitable plant growth promoting fungus for growth and establishment of tissue culture derived ginger. *Biofertiliser Newsletter*. 17 (1): 1 - 3

Taylor, J. and Harrier, L. A. 2001. A comparison of development and mineral nutrition of micropropagated *Fragaria ananassa* cv. Elvira (Strawberry) when colonised by nine species of arbuscular mycorrhizal fungi. *Appl. Soil. Ecol*. 18 (3): 205 - 215

Tepfer, D. 1984. Genetic transformation of several species of higher plants by *Agrobacterium rhizogenes*: Phenotypic consequences and sexual transmission of the transformed genotype and phenotype. *Cell*. 37: 959-967

Tepfer, D. 1990. Genetic transformation using *Agrobacterium rhizogenes*. *Physiol. Pl*. 79: 140-146

Thanuja, T. V. and Hegde, R. V. and Sreenivasa, M. N. 2002. Induction of rooting and root growth in black pepper cuttings (*Piper nigrum* L.) with arbuscular mycrorrhiza indigenous to mexico. *Scientia Horticulturae*. 92: 347 - 359

Tiwari, P. and Adholeya, A. 2002. *In vitro* co – culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T - DNA transformed roots. *FEMS Microbiol. Lett.* 206: 39 - 43

Tiwari, R. K., Trivedi, M. Guang, Z. C., Guo, G. Q. and Zheng, G. C. 2008. *Agrobacterium rhizogenes* mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots. *Biologia Plantarum* 52 (1): 26-35

Toivonen, L., Ojala, M. and Kauppinen, W. 1991. Studies on optmisation of growth and indole alkaloid production by hairy root cultures of **Catharanthus roseus**. *Biotech. Bioeng*. 37: 673 - 680

Trindade, A. V., Lins, G. M. De L. and Maia, I. C. S. 2003. Substrates and mycorrhizal fungi for micropropagation of banana seedlings in the acclimatisation phase. *Revista Brasileira de Fruticultura*. 25 (1): 137 - 142

Trypsteen, M., Van-Lijsebettens, M., Van-Severen, R. and Van-Montagu, M. 1991. *Agrobacterium rhizogenes* mediated transformation of *Echinacea purpurea*. *Pl. Cell Rep*. 10: 85 - 89

Vanhala, L., Hiltunen, R. and Oksman-Caldenty, M. 1995. Virulence of different *Agrobacterium* strains on hairy roots formation of *Hyscyamus muticus*. *Pl. Cell Rep*. 14: 236-240

Van-Wordragen, M. F., Pieter, B. F., Ouwerkerk, C. M. and Dons, A. J. M. 1992. *Agrobacterium rhizogenes* mediated induction of apparently untransformed roots and callus in Chrysanthemum. *Pl. Cell Tissue and Organ Cult*. 30:149 - 157

Varghese, S. 2006. Genetic transformation for hairy root induction and enhancement of secondary metabolites. M. Sc. Thesis. Kerala Agricultural University, Thrissur, Kerala, India. 199 p

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

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

Von, A. 1998. State of commercial use of AMF-inoculum in Germany. In: Gianinazzi, S. and Schepp, H. (eds.) Arbuscular mycorrhizas in sustainable soil-plant systems. Report of 1997 activities, Cost Action 821, Iceland. 153

Wang, J. W. and Tan, R. X. 2002. Artemisinin production in *Artemisia annua* hairy root cultures with improved growth by altering the nitrogen source in medium. *Biotech. Lett*. 24: 1153 - 1156

White, F. F. and Nester, E. W. 1980. Hairy root: Plasmid encode virulence trait in *Agrobacterium rhizogenes*. *J. Bacteriol*. 41: 1134-1141

Wilson, D. 1993. Induced mutagenesis in rose under *in vitro* and *ex vitro* culture. Ph.D. thesis, Kerala Agricultural University, Thrissur, 239 p

Wysokinska, H. and Chmiel, A. 1997. Transformed root cultures for biotechnology. *Acta Biotech*. 17: 131-159

Wyss, P. and Bonfante. 1993. Amplification of genomic DNA of arbuscular – mycorrhizal (AM) fungi by PCR using short arbitrary primers. *Mycol. Res*. 97: 1351 - 1357

Xu, T., Zhang, L., Sun, X., Zhang, H. and Tang, K. 2004. Production and analysis of organic acids in hairy root cultures of *Isatis indigotica* Fort. (Indigo wood). *Biotech Appl. Biochem*. 39: 123 - 128

Yano-Melo, A. M., Saggin, O. J. Jr., Lima-Filho, J. M., Melo, N. F. and Maia, T. C. 1999. Effect of arbuscular mycorrhizal fungi on the acclimatisation of micropropagated banana plantlets. *Mycorrhiza*. 9: 119 – 123

Yoshikawa, T. and Furuya, T. 1987. Saponin production by cultures of Panax ginseng transforemed with *Agrobacterium rhizogenes*. *Pl. Cell Rep*. 6: 449-453

Yoshimatsu, K., Shimomura, K., Yamazaki, M., Saito, K. and Kivichi, F. 2003. Transformation of Ipecac (*Cephaelis ipecacuanha*) with *Agrobacterium rhizogenes*. *Planta Med*. 69: 1018-1023

Zambryski, P. 1988. Basic process underlying *Agrobacterium* mediated DNA transfer to plant cells. *A. Rev. Genet*. 22: 1-30

Zdravkovic-Korac, S., Muhocski, Y., Druart, P., Calic, D. and Radojevic, L. 2004. *Agrobacterium rhizogenes* mediated DNA transfer to *Aesculus hippocastanum* L. and the regeneration of transformed plants. *Pl. Cell Rep*. 22: 698-704

Zeleznik, A., Barieevic, D. and Vodnik, D, 2002. Micropropagation and acclimatisation of yellow gentian. *Kmetijstvo*. 79 (1): 253 - 259

* - Oringinals not seen

Appendix

APPENDIX I

Ruakara nutrient solution

Macronutrient stock solution (A) (g l^{-1})

$Mg(NO_3)_2.6\ H_2O$	- 4.94
$Ca(NO_3)_2.4\ H_2O$	- 16.78
NH_4NO_3	- 8.48
KNO_3	- 2.28

Macronutrient stock solution (B) (g l^{-1})

KH_2PO_4	- 2.67
K_2HPO_4	- 1.64
K_2SO_4	- 6.62
Na_2SO_4	- 0.60
Nacl	- 0.33

Minor nutrients supplements (mg l^{-1})

H_3BO_3	- 128.80
$MnCl_2.\ 2\ H_2O$	- 81.10
$Zn\ Cl_2$	- 23.45
$CuCl_2.\ 2\ H_2O$	- 4.84
$(NH_4)_6\ MO_7\ O_{24}.\ 4\ H_2O$	- 0.83
Ferric citrate pentahydrate	- 809.84

The nutrient solution for plants were prepared by mixing 200 ml each of the macronutrient stock solutions with 100 ml of the micronutrient supplement and diluting to 4.5 l with deionised water.

APPENDIX II

Formalin Acetic Acid (FAA) (1000 ml)

Formalin (40 %) - 50 ml

Glacial Acetic Acid - 50 ml

Ethanol (95 %) - 900 ml

APPENDIX III

a. Tryphan blue stain

Tryphan blue - 0.05 g

Lactophenol - 100 ml

b. Lactophenol (1000 ml)

Lactic Acid - 100 ml

Phenol - 100 ml

Glycerol - 200 ml

Water - 600 ml

c. Murashige and Skoog (MS) media composition

Components	Quantity (mg l^{-1})
Major Nutrient Stock (1)	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$.2H2O	440
Mg So_4. 7 H_2O	370
KH_2PO_4	170
Miner Nutrient Stock (2)	

H_3BO_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
Stock Solution (3)	
$FeSo_4.7H_20$	27.8
$Na_2EDTA.2H_20$	37.3
Organic Constituents	
Myoinositol	100
Pyridoxine HCl	0.5
Glycine	2.0
Thiamine	0.1
Nicotinic acid	0.5
e) Sucrose	30g / l
f) Agar	8g / l

d. Yeast Extract Agar media

Yeast Extract - 1.0 g

Beef Extract - 5.0 g

Peptone - 5.0 g

$MgSO_4.7H_2O$ - 0.5 g

Sucrose - 5.0 g

Agar - 20.0 g

pH - 7.2

Distilled Water - 1000 ml

Agrobacterium rhizogenes mediated *in vitro* multiplication of AMF (*Glomus intraradices*) in *Artemisia annua* L.

By

K. THIAGARAJAN

(2006.11.111)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

VELLANIKKARA THRISSUR-680 656

KERALA, INDIA

2010

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are an important biofertiliser and biocontrol agent for agricultural crops. It plays an important role in phosphorous uptake and imparts stress tolerance in plants. At present, large scale multiplication of AMF is done in pots using a suitable host. However, it is difficult to maintain pure culture of AMF in pot culture. Moreover, *in vitro* multiplication on artificial media is not possible due to its obligate symbiotic nature.

One of the recent approaches for the mass multiplication of AMF is the Ri-T-DNA hairy root technique. AMF, if colonised on hairy roots, can be a novel technique for its *in vitro* mass multiplication. Hence, a study was undertaken to multiply AMF (*Glomus intraradices*) in the hairy roots of *Artemisia annua* under *in vitro* conditions.

The studies on the effect of *G. intraradices* on *Artemisia* confirmed root colonisation and spores in rhizosphere. Among the two strains of *Agrobacterium rhizogenes* used for hairy root induction in *Artemisia*, MTCC 532 was selected and was found to be superior to MTCC 2364. Hairy roots were induced on *A. annua* as per the procedure already standardised.

The cellulase enzyme (0.16 mg l^{-1}) in water agar media enhanced *G. intraradices* spore germination for inoculation on hairy roots. Normal procedures carried out to infect hairy roots of *Artemisia* with normal and germinated spores of AMF did not yield positive results. The hairy roots failed to proliferate further and the AMF inoculum also did not survive even after repeated attempts in different culture conditions (solid, liquid static and shaking). However, the possibility of using *A. annua* as a host for AMF (*G. intraradices*) was confirmed through pot culture experiments.

A. annua based inoculum was found superior to maize based inoculum and it recorded maximum survival per cent, number of leaves, plant height (cm), number of tillers, plant fresh weight and dry weight in pot culture experiment using tissue culture (TC) derived ginger plants.

The present study revealed the possibility of using *A. annua* as a host for multiplication of AMF. However, no root colonisation was observed on hairy roots of *A. annua*. Microbial contamination during AMF spore inoculation was the main limiting factor for proliferation. Thus, the procedure for *in vitro* inoculation of AMF spores in hairy roots of *A. annua*, are yet to be standardised. A suitable medium that will allow survival and proliferation of hairy roots and AMF together also needs to be standardised.