ROOT ORGAN CULTURE OF VESICULAR ARBUSCULAR MYCORRHIZAL FUNGI

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CERTIFICATE

This is to certify that the thesis entitled **"ROOT ORGAN CULTURE OF VESICULAR ARBUSCULAR MYCORRHIZAL FUNGI"** submitted by **Mr.D. DAYALADOSS,** in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN AGRICULTURAL MICROBIOLOGY**, to the University of Agricultural Sciences, Bangalore is a record of research work carried out by him, under my guidance and supervision and that no part of the thesis has been submitted for the award of any degree, diploma, associateship, fellowship or similar titles.

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INTRODUCTION

I INTRODUCTION

Vesicular-arbuscular mycorrhizal (VAM) fungi are beneficial symbiotic microorganisms that colonize the root and increase the growth and yield of most crop plants. The improved plant growth is attributed to increased nutrient uptake, production of growth promoting substances, tolerance to drought, salinity and transport shock, resistance to plant pathogens and synergistic interactions with beneficial soil microorganisms like nitrogen fixers, phosphate solubilizers etc. However, harnessing these beneficial effects for commercial utilization has proven difficult since VAM fungi are obligate symbionts and cannot be cultured on laboratory media.

Several attempts have been made to culture these fungi on artificial media with little or no success. Mosse in 1962 established that surface sterilized spores of VAM fungi can be used to infect plants under axenic conditions. Since a single spore can successfully infect a plant, this provided a way of starting a pure line of a VAM fungus by transplanting the infected seedlings to a soil or sand medium and allowing new spores to form.

The obligate symbiotic nature of VAM fungi presently dictates that all VAM inoculum must be grown on roots of an appropriate host plant. Although hydroponic culture system and axenic nutrient techniques have been suggested to raise mycorrhizal inocula they have not become very popular.

Agrobacterium rhizogenes is a soil-borne pathogen causing "hairy root" in dicotyledonous plants. When wounded tissues are infected with the bacteria, they form large number of hairy roots. These are genetically transformed hairy root produced by the Ri - plasmid of *Agrobacterium rhizogenes*. The transformed roots can be subcultured as exercised roots. The desirable characteristic of these transformed roots is their ability to quickly form numerous lateral roots. Tepfer (1984) concluded that they are better adapted to growth in culture than normal roots and that they also survive longer period without subculture.

Such root organ cultures have been used to culture VAM fungi. Some studies have been conducted to continuously maintain VAM fungi in root organ culture for a long time. The present investigation was undertaken to find out the possibilities of improving the method used for root organ culture and co-culturing VAM fungi in it. Thus, the objectives of the present investigation are :

- Development of a suitable protocol for the production of hairy roots.
- Development of a suitable method for axenic cultivation of VAM fungi and hairy roots.

REVIEW OF LITERATURE

II REVIEW OF LITERATURE

Mycorrhiza :

Mycorrhiza literally meaning "Fungus root" was coined by A.B. Frank in 1885. This beneficial mutualistic association is highly prevalent in most crop plants. They are universally present in all soils in association with a great variety of plants of different taxonomic groups (Nicolson, 1967, Gerdeman, 1968).

There are two types of mycorrhiza, Ectomycorrhiza and Endomycorrhiza. In ecotomycorrhiza, the fungus grows around the root surface in the form of fungal mantle and inter-cellularly in the root cortex and epidermis to form a distinct 'Hartig net'. Ectomycorrhizal associations are commonly found in the temperate forest trees. In endomycorrhiza, fungus mainly grows inside the root, inter and intra-cellularly. The different types of endomycorrhiza are as follows, arbutoid, monotropoid, ericoid, orchid and the most common vesicular arbuscular (VA) mycorrhiza. VA mycorrhizal fungi produce the fungal structures, vesicles and arbuscules in cortical roots (Bowen, 1987). Arbuscules are specialized, highly branched fungal hyphae which serve as structures of nutrient exchange in mutual association. These are formed by the repeated branching of the arbuscular trunk giving rise to fine dichotomously branched filaments which occupy the entire lumen of the cell. The life span of the arbuscules is limited to few days (4 to 5 days) and later absorbed by host system (Cox and Tinker, 1976).

All VAM fungi belong to the genera *Glomus, Gigaspora, Scutellospora, Acaulospora, Entrophospora* and *Sclerocystis* and they form arbuscules. Vesicles are terminal globose bodies that develop by an intercalary or terminal swelling of the VAM hyphae in the root cortex. They are inter-cellular or intra-cellular and differ in size, shape, wall structure, number and content according to the endophyte involved. The vesicle number was high in *Glomus fasciculatum* and *G. monosporum* and *G. caledonicum* (Abbott and Robson, 1981). Some species of VAM fungi produce thin-walled vesicles, which function as storage organs (McLennan, 1976), whereas thick-walled may function on resting spores (Gerdemann, 1974).

VAM fungal hyphae spread in cortical cells inter and intra-cellularly. This inter and intra-cellular spread in cortical cells has little impact on gross morphology of roots, but has significant change in root anatomy and in particular on plant physiological properties (Lindermann, 1988).

Taxonomy and occurrence of VAM :

Recent classification described VAM-fungi under a new order Glomales (Morton and Benny, 1990). The order Glomales has two sub orders Glominae and Gigasporinaeae. Under sub order Glominae, all the four arbuscular fungi which form intra radical vesicles have been placed. These four genera include *Glomus, Sclerocystis*, *Entrophosphora* and *Acualospora*. *Gigaspora* and *Scutellospora*

which are grouped under sub order Gigasporinaeae form extra radical auxillary cells and produce no intraradical vesicles. Order Glomales belongs to class Zygomycetes of the sub-division Zygomycotina and division Amastigomycotina.

The first report of VAM occurrence was in popular roots by Dangeard (1900) which he named as *Rhizophagus poplinus* illustrated by arbuscules, vesicles and multinucleate hyphae. VAM probably evolved with the Devonian land flora (Nicolson, 1975) which now occurs in all natural ecosystems of all vascular plants forming mutualistic association (Trappe, 1987). The discovery of arbuscules in Aglaophytan and in the plants of Rhynie chert major, an early Devonian plants provides major evidence to the establishment by VAM association as early as 400 million years ago (Taylor *et al.*, 1995). Few angiosperm families are constantly non-mycorrhizal which includes Cruciferae, Chenopodiaceae, Caryophyllaceae and Cyperaceae (Bowen, 1987).

Occurrence of VAM fungi in all types of soils, climates and in extreme environments emphasizes its dominant role in crop plant mutualistic association. They occur in wide ecological range from aquatic to the desert environment, plants growing in Antarctica (Demars, 1995) and in temperate and tropical regions (Mosse *et al.*, 1981). Several reports confirm their occurrence in desert environment, coal mines (Mathur and Vyas, 1994) and also in sand dunes (Koske and Gemma 1996). This wide spread occurrence of VAM is seen in all communities of plants with different intensity.

Distribution and Impact of VAM on Plant growth :

VAM fungi are the most widespread in their distribution geographically and among plant species. They are believed to be disseminated intercontinentally prior to continental drift as supported by fossil records of earlier plants (Berch, 1986, Remy, 1994 Hass *et al.* 1994). They occur more frequently in cultivated top soils and most economically important crop plants (Menge, 1984, Bagyaraj, 1991).

The mycorrhizae represents one of the nature's best gifts to mankind in conversion of arid soil to fertile and productive soil (Mukerji and Dixon, 1992). The increase in effective nutrient absorbing surface provided by VAM fungi is primarily responsible for the increase in uptake of soil nutrients by plants. The potential use of VAM fungi in increasing crop yield has attracted much attention. Controlled green house studies have demonstrated that VAM fungi improve crop growth and yield by several mechanisms (Powell and Bagyaraj, 1984). The benefits of association improves uptake of macro and micro nutrients, increased tolerance to abiotic and biotic factors and change in the production of growth regulators and their physiological implications on plant growth (Jarstfer and Sylvia, 1992).

VAM in nutrition uptake of phosphorus :

Inoculation with VAM fungi is known to enhance plant growth and mineral uptake. An increased plant growth by VAM fungal inoculation has been reported by

several workers on legumes, field crops and horticultural crops (Pairunan *et al.*, 1980, Lu and Miller 1989, Boaon *et al.*, 1993, Vinayak and Bagyaraj, 1990).

Much of the work has been carried out on the availability of phosphorus (Mosse, 1973). The effectiveness of mycorrhizae in phosphorus nutrition of plant is related to the ability of fungus to colonize and provide an extensive media for hyphal absorption and translocation of P through over considerable distance (Smith and Gianinazzi-Pearson, 1988). Hyphae can readily absorb even in high P fixing soils. Mycorrhizal inoculation along with rock phosphate significantly increased the uptake of P, Cu, Zn and dry mater yield of *Leucaena leucocephala* plants (Manjunath *et al.*, 1989). This process of P absorption was also studied in ^{32}P labelled inositol and phosphatase activity in mycorrhizal roots suggesting the potentiality of VAM fungi in efficient translocation of P in gray birch and red maple roots (Antibus *et al.*, 1997).

Nitrogen and carbon nutrition :

Mycorrhizal association enhances N gain by increasing N fixation rates of plant nitrogen fixing bacterial association. Increase in the rates of N fixation with mycorrhizae have been recorded in legumes (Hayman, 1987). Increased tissue P and N concentration with *Rhizobium* and VAM inoculation was also reported by Azcon and Barea (1992), in legume *Medicago sativa.* It has been demonstrated that N fixed in association with one plant can be transported to an adjacent, non-nitrogen

fixing plant through the mycorrhizal fungal hyphae. Van Kessel *et al.* (1985) showed that N fixed by soybeans was transported to maize through VA mycorrhizae and significantly increased the growth and N status of maize plant. Frey and Schuepp (1993) demonstrated the transfer of N from *Trifolium alexandrium* to associated non-legume (*Zea mays*) through VA mycorrhizal hyphae using ^{15}N tracer techniques.

Mycorrhizal roots are often distributed in places where decomposing tissues are most prevalent. Higher concentration of mycorrhizal hyphae in decomposing litter was observed than in the surrounding soil matrix in both tropical and Arid zones (Bansal and Mukerji, 1994b, Kapoor and Mukerji 1992).

Trace elements and other attributes :

Mycorrhiza facilitates uptake of other slowly diffusing nutrients such as Zn, Ca, Cu, K, Mg and Mn and these may also attribute for drought resistance to host plant (Hall, 1978 ; Azcon and Ocampo, 1981 ; Pai *et al.*, 1994 ; Sylvia *et al.*, 1993 ; Kothari *et al.*, 1990).

In addition to nutrient uptake, mycorrhizae also enhance the transport of water from soil to plant. Since mycorrhizal plants have higher transportation rates, the water uptake per unit root length and thus the mass flow of soil solution to the root surface is about twice as high as non-mycorrhizal plants

(Allen and Allen, 1986). The increased water gain by the mycorrhizal plant enhanced its ability to survive water stress (Nelson, 1987).

VAM inoculum production :

The obligate symbiotic nature and inability to grow VAM in pure culture necessiates inoculum production in an appropriate host plant. This provoked researchers to develop methods for maintaining and mass producing VAM fungi incorporating all the factors required for growth.

Soil Pot Culture :

Cultures of VAM fungi on plants growing in disinfested soil have been the frequent and common method used for producing inoculum. The production of soil based inoculum is relatively easy and a well established technique (Menge, 1984). A highly susceptible host plant should be used and should produce abundant roots required for the fungus to infect and reproduce. Trap plants should be screened to insure that maximum levels of inoculum are produced. Quite a large number of host plants have been tested for VAM inoculum production (Bagyaraj and Manjunath, 1980). Sreenivas and Bagyaraj (1988a) reported Rhodes grass (*Choris gayana*) to be the best host for mass multiplication of *Glomus fasciculatum*. The host plant which can be propagated from seeds are preferable to that of cuttings since seeds can be more easily disinfected than cutting. All the components of the culture system should be disinfected before the initiation of the process. The purpose of soil

disinfestation is to kill existing VAM fungi, pathogenic microorganisms and weed seeds. Pasteurization of soil by heating to a higher temperature (Fry, 1982) and by using aerating steam with different time interval was proposed by Sylvia and Jarstfer (1994). Some other methods of disinfecting soil includes fumigation with ethylene bromide, autoclaving or use of ionizing radiations (Thompson, 1990 ; Jensen, 1983).

Soil inoculum from pot culture of many VAM fungi may be stored in polythelene bags for many months. Feldmann and Idczak (1992) found that the infectivity of *G. etunicatum* inoculum which was stored at 20-23^0 C and 30-50% relative humidity for 3 years was reduced by only 10-15%. However, the more relative storage of a wide range of VAM fungi can be by cryopreservation at frozen temperature of -70^0 C (Douds and Schenck, 1990). The culture of VAM as soil inoculum has been most commonly used though more serious problem being maintenance of high inoculum potential and airborne pathogens and pests. The soil inoculum being heavy there is problem in transportation and distribution (Sieverding, 1991).

Soilless media for pot culture :

Culturing VAM fungi in soilless media (artificial substrates) avoids the need for sterilization and other drawbacks in soil culture methods. The soilless mixture should have good water holding capacity for plant growth with good aeration. Different substrates are used like bark, calcined clay, expanded clay, perlite, peat

and vermiculite. Dehne and Beckhaus (1986) used expanded clay as carrier material for VAM fungi and were successful in colonization of tropical perennial crops (Feldmann and Idczak, 1992). Sreenivasa and Bagyaraj (1988b) found that 1:1 perlite : solirite is the best substrate for mass multiplication of *G. fasciculatum*. Jarstfer and Sylvia (1992) cultured 15 species of VAM fungi of *Acaulospora, Gigaspora, Glomus* and *Scutellospora* in different soilless media.

Aeroponic and hydroponic systems for VAM inoculum production :

A culture system which has a fine mist of defined nutrient solution to the roots of host plant is termed as aeroponic culture (Zobel *et al.*, 1976). Aeroponic culture system was adopted for growing VAM-fungi by Hung and Sylvia (1988). Plants were first grown in sand or vermiculite with selected VAM inoculum before they were transferred to this system. Aeroponic cultures have produced higher spore concentration than that of soil based pot cultures. The inoculum produced can be stored for longer time at low temperature of 4^0 C. Since no substrate is present with the inoculum, with aeroponic culture of roots, it is possible to produce hundreds of thousands of propagules per gram dry weight of root (Sylvia and Jarstfer, 1992). However, drying of the roots reduces the infectivity of the inoculum. Therefore transportation and commercialization would be a problem. Moreover, high quantities of infected roots were needed to compete with indigenous VAM fungi.

In hydroponic culture system inoculum can be produced using the nutrient film techniques (NFT). It is a modified culture system in which the plant roots grow

in a shallow layer of flowing nutrient solution which is circulated by a pump. Mosse and Thompson (1984), used the same method to produce VAM inoculum using beans (*Phaseolus vulgaris*) as host plant. The inoculum produced consisted of root mat of 10 to 15 mm thick with extensive fungal mycelium and spores on the surface of the roots (Warner *et al.*, 1985). However, the disadvantages of aeroponic culture applies to this VAM root inocula also.

Hawkins and George (1997) cultured mycorrhizal fungus *Glomus mosseae* with three host plants *Linum usitatissimum*, *Sorghum bicolor* and *Triticum aestivum*. The root colonization percentages were within the range of colonization rates obtained with solid substrate culture, and viability of mycorrhizal structures were also similar to other inocula. Hydroponic culture so described can produce high mycorrhizal colonization under short culture period.

Axenic culture of VAM fungi :

The literature on *in vitro* cultivation of VAM fungi are increasing due to the importance of such study for the potential use of this associate symbiont in the field of agriculture. An attempt has been made to review the different *in vitro* culturing techniques of VAM fungi. The study is focused on Ri - plasmid transformed root cultures offering an efficient method for growing VAM fungal colonized roots.

Tissue culture techniques :

Plant tissue culture is the science and art of growing isolated plant cells, tissues, organs or whole plants, on semisolid or liquid synthetic nutrient media, under aseptic and controlled environments.

Plant tissue culture techniques can be initiated from unorganized tissues or organized structures. The first category includes callus cultures, cell suspension cultures and protoplast cultures. Second category includes well recognized methods of tissue culture like meristem culture, shoot tip or node cultures, embryo culture and isolated root cultures.

Root cultures :

Robbins (1922) postulated that true *in vitro* cultures could be raised from meristematic cells of root tips and stem tips and these root cultures are useful in production of secondary metabolites and in basic studies on mycorrhizal associations.

Rajanasiriwong *et al.* (1987) proposed the idea of tissue culture for the mass production of VAM fungi. They described that mycorrhizal colonization was maximum in liquid agitated culture than on agar. By agitation, contact between spores and root surfaces has increased.

Wood (1987) developed an axenic production system based on the root organ culture techniques of Mosse and Hepper (1975). The system utilizes stable clones of *Trifolium incarnatum* roots grown in porous vermiculite saturated with a modified Murashige and Skoog (1962) culture medium.

Genetic transformation methods :

Plant genetic engineering is an off-shoot of many remarkable developments in the present century witnessed in the field of plant science. Conventionally gene transfer for the improvement of plant species is achieved by hybridization. For this, concurrent flowering in both the parental species is a primary requirement. In some species it is not possible and also in some other species with genetic incompatibility. Presently many novel gene transfer systems are available for the development of transgenic plants and few of them are summarized below.

Microinjection method :

This technology has been found to be very useful for genetic transformation of animals (Capecchi, 1980), while its application to plants is limited. Griesbach (1987) could produce transgenic *Petunia hybrida* plants by microinjecting chromosomes of *Petunia alpicola.* Neuhaus *et al.* (1987) microinjected embryoids (haploids) using microscope and NPT-II marker gene and succeeded in raising 51% transformed plants.

Microprojectile bombardment Mediated transformation :

Klein *et al.* (1987) for the first time reported DNA delivery into living plant cells through microprojectile bombardment. Using 0.22 caliber cartridge, tungsten microprojectiles carrying DNA were propelled, through a vacuum chamber into living cells. By this method successful transformation was obtained first in tobacco. Reports were pouring in after this by different scientists. (Formm *et al.*, 1990 ; Songstad *et al.*, 1992 ; Koziel *et al.*, 1993).

Bidney *et al.* (1992) used microprojectile bombardment to enhance the rate of *Agrobacterium* infection in sunflower. They bombarded the shoot pieces with microprojectile (without DNA) to achieve efficient and uniform wounding before co-cultivation with *Agrobacterium.*

Polyethylene glycol (PEG) mediated transformation :

In this method, protoplasts are treated with polyethylene glycol (PEG) to alter the plasma membrane properties and obtain permeability. This enables the entry of exogenous macromolecules in to the cytoplasm. Krens *et al.* (1982) for the first time reported transfer and expression of *Agrobacterium tumefaciens* T-DNA genes into tobacco protoplast using PEG treatment.

Electroporation mediated transformation :

In this method protoplasts are subjected to electric pulses at high field strength, which causes reversible permeabilization of the plasma membrane which

enables the entry of DNA. This method has been successful in maize (Rhodes *et al.* 1988), rice (Shimamoto *et al.* 1989) and in orchids (Griesbach and Hamond, 1993).

Agrobacterium rhizogenes mediated gene transfer :

Agrobacterium rhizogenes mediated gene transfer in the novel gene transfer method among all methods described earlier. This was the opening of an era of transgenic plants. Initially successes were limited to members of only one plant family *Solanaceae*. However, over the past few years, transformation has been achieved in a wide range of plant species, including many agronomically important ones (Lindsey, 1992).

The first transgenic plants were produced through *Agrobacterium* mediated transformation of *Nicotiana tobacum* leaf explants (Horsch *et al.*, 1985 ; DeBlock *et al.*, 1984). Since then many plant species have been genetically engineered by various techniques.

Ri T-DNA transformed roots :

Agrobacterium rhizogenes is responsible for the hairy root syndrome of dicotyledonous plants. These bring about abundant proliferation of roots at the wounded site of bacterial infection. Hairy roots contain a portion (T-DNA) of a large bacterial plasmid (Ri - plasmid), which directs the growth and differentiation of the transformed plant cells (Chilton *et al.* 1982).

Hairy roots grow very actively *in vitro* in the absence of hormones with a characteristic highly branched and plagiotropic pattern (David *et al.*, 1984). These hairy roots induced by *Agrobacterium rhizogenes* synthesize specific opines of which three major types have been so far identified, agropine, mannopine and cucumopine (Petit and Tempe, 1985). These opines are produced corresponding to different families of Ri - plasmids, like mannopine Ri and agropine Ri - plasmids (Costantino *et al.*, 1981). The root formation by agropine Ri strains are usually more abundant than that by mannopine Ri strains (Petit *et al.*, 1983).

The virulent strains of *Agrobacterium* harbour a large (150-200 kb) Ti and Ri - plasmids which confer a number of properties to the organism. These plasmids are responsible for the DNA transfer and integration. Genetic and molecular analysis showed that both the plasmids contain two regions, T-DNA (transferred DNA) region that are transferred to the plant and the vir (virulence) region which determines the system by which the T-DNA in transported and are not themselves transferred during infection. The combination of T-region and Vir-region determines the host range properties of the *Agrobacterium*. The T-DNA region are flanked by border sequences called the right and left borders (24 bp repeats) between which lies the T-DNA. The T-DNA contains eight to thirteen genes (Nester *et al.*, 1984) which include a set of genes for the production of phytohormones, genes which code for opines (sugar amino acid condensation products) and a gene which modulates the expression of the phytohormone coding genes.

The initial studies have confirmed that any foreign DNA can be transferred to plant along with the existing T-DNA genes. After this discovery, efficient plant transformation and transfer of DNA of interest could be possible by constructing transformation vectors by removing phytohormone biosynthetic gene from the T-DNA region. Thus, vector and binary vectors have been constructed for transformation studies.

Initially, transformation studies were aimed for development of transgenic plants related to control of insects, weeds and plant pathogens. Transgenic approach has also been extensively used to understand different metabolic processes either by over expressing or reducing the expression of target gene. The other aspect of transformation in development of hairy roots by Ri – plasmid induction process and metabolism remains same. The targetted extensive proliferation of hairy roots by this process, can be successfully used in extraction of metabolites produced in root system and for the establishment and growth of obligate mycotrophs like VA mycorrhizal fungi.

Transformation by *A. rhizogenes* in some crop plants :

The natural and effective way of transformation in most of the dicots by *A. rhizogenes* is by Ri T-DNA. This has been successfully done in several horticultural, medicinal and aromatic crops and in some trees species which are difficult to propagate.

Toyoda (1993) reported transformation process in barley. Surface sterilized germinated seedlings were used for the study. By this method some of the variations brought about due to plant pathogen interaction were studied. Transformation in chickpea was carried out by Riazuddin and Husnain (1993). Four genotypes of chickpea were found to be susceptible for both the strains of *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*. Root induction and *Agrobacterium* based gene transfer were studied. This mode of transformation could be potentially useful in breeding resistant varieties.

Transformation of potato using mannopine and cucumopine strains of *A. rhizogenes* with two cultivars was done by Dobigny *et al.* (1996). An overnight treatment of stem segments with NAA was necessary to induce root formation. When explants from potato were pretreated with 26.5 µM NAA, 71.9% produced roots. Most of the transformed roots were highly branched and grew rapidly, compared to non-transformed roots with no branching and poor growth.

Direct regeneration of *A. rhizogenes* transformed plants from stem segments of potato was reported by Dobigny *et al.* (1996). The transformed roots were easily regenerated to shoots and the frequency of transformation was also high. Histological study revealed that the transformed shoots developed directly from cells of the internode sections.

A similar work was also done on different crops like cucumber (Toppi, *et al.,* 1997) and Brassica (Henzi, 1999).

Establishment of grape vine root lines were better with transformed explants using *A. rhizogenes* (Gribaudo *et al.*,1995). Different explants like stem, internode, petioles without leaf blade were used for the study. Earlier, Lupo *et al.* (1994) used *Agrobacterium rhizogenes* transformed plant roots as a source of grape vine viruses for purification and inoculum.

Transformed grapevine roots contained as much viruses as non-transformed roots and more than leaves, as assessed by ELISA and thin sectioning. All viruses multiplied and persisted in root cultures, were successfully used for purification. Torregrosa and Bouquet (1997) co-transformed both *A. rhizogenes* and *A. tumefaciens* with plasmids carrying gene encoding for the grape vine chrome mosaic virus coat protein.

Stylosanthes humilis is one of the most readily transformed legume (Elliott and Manners, 1993); it represents a useful model system for investigating the improvement of pasture legume. *Agrobacterium* mediated transformation proved to be useful technology for developing fungal disease resistance and for improvement of digestibility. Tabaeizadeh (1993) used *A. rhizogenes* as a better tool for the transformation of the legume Bird-foot trefoil, (*Lotus corniculatus*). The results encouraged the use of *A. rhizogenes* as transformation agent instead of

A. tumefaciens. The incidence of transformation was high and Ri T-DNA remains unmodified through regeneration.

Medicinal and alkaloid production :

Kazuki Satto (1993) successfully co-cultured *Digitalis purpurea* L. (Foxglove) which is one of the most commonly used medicinal plants in the world, a source of cardioactive glucoside, digitoxin, gitoxin etc. *Agrobacterium* Ri - plasmid induced hairy roots were used for the multiplication of plants. This method was found to be suitable for the genetic manipulation of the metabolism of rapidly growing hairy roots which produce more quantity of secondary products.

Recently focus is on transformation of medicinal plants to produce pharmacologically useful alkaloids and commercially important trees which are difficult to establish.

The medicinal plant *Rubia peregrina* L. is known for production of anthraquinone and alizarin. Lodhi and Charlwood (1996) had co-cultured *A. rhizogenes* (LBA - 9402) harbouring the binary vector pMON 9703 *in vitro*. The total anthraquinone and alizarin contents of transgenic root cultures were measured by spectrophometry and HPLC. Two fold higher anthraquinone and 3 fold higher alizarin in transformed roots than in field grown roots was recorded.

Genetic transformation of gentian plants revealed a stable integration of transferred DNA by *A. rhizogenes* A_4 harbouring root inducing plasmid (pRi A_4). Transgenic plants were regenerated from the hairy roots at a frequency of 19% on MS medium (Hosokawa *et al.*, 1997).

Hairy root technology is of greater importance for most of the valuable alkaloid extracts from the roots. The alkaloid production was also high in these transformed roots. Yoshihito *et al.* (1993) compared the alkaloid production of *Atropa belladonna* cell cultures with that of hairy root cultures. The principle alkaloid hyoscyamin and scopolamine reached 0.68% of dry weight which is 1.6 and 2.6 times higher than normal roots. Mukerjee *et al.* (1995) cultured *Artemisia annua* with different strains of *A. rhizogenes* and hairy roots were grown without growth regulators and recovered high levels of benzyladenine (25-30 mg/l). Alkaloid production was also increased by 1.5 mg/g dry weight in hairy roots of *Catharanthus roseus* as reported by Jung *et al.* (1995).

Recently, Nassbaumer *et al.* (1998) published higher biosynthesis of alkaloids in *Datura candida* transformed root clones. Among the three basal media tested, half strength Gamborg's B5 performed better with 5% sucrose (288 mg/dry wt./flask). Addition of exogenous nitrate increased the hyoscyamine content in hairy roots (0.54% dry wt.) but scopolamine content was significantly reduced. Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures was by genetic engineering (Jouhikainen *et al.*, 1999). This plant was

chosen for its capacity to produce very high amounts of tropan alkaloids (up to 6% dry weight in the leaves of mature plant). A great variation was observed in the tropan production among 43 positive transformants. The best clone (KB7) produced 17 mg/l of scopolamine which is 100 times more than that of control clones.

Transformed organ cultures of *Withania somnifera* was established following infection with wild type strains of *Agrobacterium* (Swagata and Sumita, 1999). The transformed and regenerated plants were able to synthesize both the major withanolides A and D. The withanolide synthesis was much higher (0.07-0.1% withaferin - A and 0.085-0.025% withanolide - D) than in non-transformed shoot cultures.

Regeneration Woody plants through *A. rhizogenes* has been reported with *Rauvolfia serpentina* (Benjamin, 1993), black locust, (Han *et al.*, 1993) and coast red wood (*Sequotia sempervirens*) (Mihaljevic *et al.* 1999). Das *et al.* (1996) studied rooting of cashewnut trees with stem tips and cotyledons. Nearly 96.8% rooting was observed when shoot tips were treated with *A. rhizogenes*.

Hairy root resulting from infection by *A. rhizogenes* from thirty plant species have been published by Mugnieer (1988). Hairy roots have been cultured for at least 2 to 6 years on MS medium. Some of the hairy root cultures were spontaneously regenerated to whole plants. Overall, host range and its implications by infection with *Agrobacterium rhizogenes* was published by Porter (1991). This

complete compilation includes about 645 sub generic taxa, including 183 named varieties, 5 classes and 2 divisions.

Establishment of VAM fungi in Ri T-DNA transformed roots :

Culturing of VA mycorrhizal endophytes has frequently been attempted but with no success. Mosse (1962) was the first to establish that surface sterilized resting spores of VAM fungi could infect plants under axenic conditions. This was the essential evidence that the spores associated with infected roots under natural conditions could initiate new symbiosis in the absence of any other microorganism. Hepper (1981) gave the evidence, that a single spore can successfully infect a plant. This provided a new era in starting a pure line of VAM fungus. Some studies relating to 'independent' growth of VA endophytes in dual culture with Ri T-DNA was first published by Mosse (1987). The rationale of this approach to the culture of VA endophyte is discussed.

The early events of VAM establishment on Ri T-DNA transformed roots of *Convolvulus sepium* was done by Mugnier and Mosse (1987), where they used the VAM fungus *Glomus mosseae* to infect the cloned roots for colonization.

Becard and Fortin (1988) developed Ri T-DNA transformed roots and used it for the establishment of mycorrhiza by *Gigaspora margarita*. Further, different aspects of root organ culture techniques proposed by Becard and Piche (1992), made

it possible to obtain VAM on transformed as well as on non-transformed roots. These methods suggest the possible use of this technique for studying complete life cycle and molecular biology of VAM fungi.

Various culture media have been used recently for growing such Ri T-DNA transformed roots viz., MS medium (Murashige and Skoog) (Mugnier, 1988), Gamborg B_5 medium (Parr *et al.*, 1988), White's medium and White's modified medium (Becard and Fortin, 1988). The last medium was preferred to MS even when diluted, because it allowed better growth of roots.

VAM spore isolation and sterilization :

Nature of VAM spore and storage :

A successful completion of the association primarily is based on pure, disease free VAM spores. Different forms of inocula have been used to form VA mycorrhiza with root organ culture. One should select those species for which spore production, sterilization and germination can be easily obtained in *in vitro* cultures. Sporocarpic chlamydospores of *Glomus mosseae* was used by Mugnier and Mosse, 1987, non-sporocarpic azygospores of *Gigaspoora margarita* and chlamydospores of *Glomus intraradices* were used by Miller – Wideman and Watrud (1984), Becard and Fortin (1988) and Becard and Piche (1990).

Based on the inoculum potential, the number of spores selected for each VAM fungus varies. The observed efficiency of species may be attributed to varied

inoculum dosage. Porter (1979) and Powell (1976) have used the most probable number (MPN) method to estimate the number of infective VA mycorrhizal propagules in unsterile soils.

Daniel *et al.* (1981) has compared the inoculum potential of spores of six VAM fungi. Spores of *Glomus mosseae* had the highest inoculum potential while, *G. constrictus, G. fasciculatum, G. epigaeus* spores had the least. The rate of increase of infection by the six VAM fungi differed more when equal spore numbers were used as inoculum than when equalized inoculum potentials were used.

Powell (1976) described that the laminate, yellow vacuolated and honey coloured spores of *Endogone* germinate quickly and consistently on agar slide buried in the soil. For germination studies spores with thick yellow walls with vacuolated contents were recommended rather than opaque spores.

Daniels and Graham (1976) observed a marked difference in germinability of aged and fresh spores of *G. mosseae*. Aged spores germinated rapidly than fresh spores. The viability of spores as determined by their germination remained constantly higher up to 6 to 8 months and there after many spores lost their bright yellow colour and glittering smooth appearance and failed to germinate (Koske, 1981).

Louis and Lim (1988a) reported that dry storage of the inoculum for 6 months at 25 to 30^0 C increased the percentage of germination of *Glomus clarum* on water agar. The germination was further enhanced, if the dry stored spores were extracted and then stored at 4^0 C for at least 2 weeks.

Surface sterilization and conservation of VAM spores :

Most important factor in establishment of axenic culture, is introducing a surface sterilized, pathogen free propagule as starting inoculum. Most of the contaminants are through the spores. The hypochlorite treatment sometimes inhibits VAM spore germination.

The chloromine – T (2%) solution with traces of surfactant and antibiotics method developed by Mosse (1962) and adopted by other workers resulted in only 32 to 68% axenic spores (Mertz Jr. *et al.*, 1979). Surface disinfestation treatment of *Glomus versiforme* and *Glomus epigaeum* with 0.05% sodium hypochlorite solution for 5 minutes done twice failed to remove all microorganisms completely from spores (Mayo *et al.*, 1986).

Treatment with hypochlorite (5%) for 5 minutes at 30^0 C was effective for surface sterilizing *Glomus caledonicum* spores from pot cultures without loosing viability, where as *A. laevis* required treatment of 1 to 2% hypochlorite for 20 minutes to ensure sterility. Hence, it is advisable to test the spore sterilizing

agent with the fungus which is being used. Since the sterilization process may affect metabolic processes in the spores, use of lowest concentration and shortest exposure time was preferred (Hepper, 1984).

VAM Spore Isolation :

The VAM spores to be sterilized must be purified as much as possible since most of the contamination is from soil, old spores or other debris. Therefore the spore extraction from soil by wet sieving and decantation (Gerdemann and Nicolson 1963) should be followed by density gradient centrifugation. By this gravitational force most of the heavier particles, debris and other materials are separated. This method of spore isolation greatly helps in complete sterilization of VAM spores. The centrifugation method followed by Furlan *et al.* (1980) has been effectively used for various genera of VAM fungi.

VAM spore germination and other factors influencing germination :

Growth of VAM spores on agar medium was observed by Gerdemann (1955). The growth was sparse and limited to only 22 mm length even after transferring to fresh agar plates. The hyphal tips, portion of hyphae and germinating spores did not grow further but the plates got contaminated (Godfrey, 1957).

Spore germination of *Gigaspora gigantea* took place very quickly with germ tube appearing in a day after the incubation. The germ tubes rapidly grew to a

distance up to 2 cm from the spore and nearly 2 to 3 germ tubes were frequently observed from a single spore (Koske 1981a). Sequiera *et al.*, (1982) reported that surface sterilized azygospores of *G. margarita* germinated readily within 3 to 5 days on all the agar media and germ tube emerged directly through the spore wall near the hyphal attachment. The germ tube grew up to 5.5 cm on the agar medium.

The regeneration of externally formed spore masses of *Glomus intraradices* tends to occur by the normal spore germination or by fine hyphae growing from the existing hyphae. The growth of main germ tubes extended up to 5 cm over a period of 2-3 weeks (Mosse, 1987). The germination process of *G. clarum* was studied by Louis and Lim (1988a and 1988b). The germ tube was produced 3 days after incubation. New hyphae extended up to 8 mm after 10 days. The growth is mainly from the subtending hyphae and also directly through the spore coat. With longer periods of incubation, the hyphae extended up to 20 mm with frequent branching. After incubation for more than 45 days, sporulation was observed on agar. These spores were spherical to globose measuring 15-25 µm in diameter. These spores were comparable to the spores produced in pot culture, being similar in structure through smaller in size.

Several studies also indicate that presence of soil microorganisms help spore germination. Mosse (1959) reported that germination of spore of *Endogone* sp. increased on water agar when non sterile soil was added. This suggested that soil microorganisms produce certain water soluble, dialysable compounds which induce

and help spore germination. High level of spore germination (65 to 80 %) of *Glomus epigaeus* occurred in unsterilized soils. Treatment of soils by autoclaving, steaming or gamma irradication totally inhibited spore germination (Daniel and Trappe 1980).

Bacteria on spore surface helped spore germination while on addition of sterilized soil extracts reduced spore germination. Further, fungal contaminants did not over come this blockage of germination (Tommerup, 1985). Azcon *et al.* in 1986, studied the effect of soil bacteria and fungi on per cent spore germination and mycelial growth of *Glomus mosseae*. All the tested microorganisms helped in the enhancement of mycelium. In contrast to above finding fungi acted as better stimulant than bacteria and also helped in the production of vegetative spores which other wise were not produced in the absence of fungi.

Mayo *et al.* (1986) observed that the percentage of germination was higher (40 to 70%) for spores surrounded by bacterial growth in *Glomus versiforme*. The surface disinfected spores showed only 3 to 26% germination. Thus, there was a drastic reduction in spore germination without these associative bacteria, leading to the conclusion that these associative bacteria help to stimulate germination. In continuation, germination of surface disinfected spores were increased by 1.5 to 2.2 fold by addition of bacteria previously isolated.

Spore germination of *Glomus fasciculatum* was stimulated by cell free extracts of non-symbiotic nitrogen fixers. Extracts of non-nitrogen fixing bacteria

did not stimulate the significant increase in spore germination except *Psendomonas putida* and *P. fluorescence* (Tilak *et al.*, 1990). By double layer water agar method, spore germination of *Gigaspora margarita* and *Glomus mosseae* was studied with *Streptomyces orientalis* suspended in the bottom layer. This stimulation was caused by volatile compounds produced by associative actinomycetes. Germination of *Glomus mosseae* was also stimulated by *Streptomyces avermitilis* and *S. griseus* and was poor on water agar without these streptomycetes colonies (Tylka *et al.*, 1991).

Establishment of dual culture :

VAM fungal infections in root organ culture was reported by Mosse and Hepper (1975). After infection the external mycelium always grew vigorously, branching quite profusely and producing many small, round vegetative spores. Dual cultures of root and VAM fungi are generally established on solid media. Adjusting the appropriate medium for co-cultivating the two organisms, the roots and the fungus, is the most important factor for successful VAM formation in root organ cultures.

Culture media for dual establishment :

A medium for co-culturing should satisfy both the requirement, the requirements of actively growing roots, which need a rich complex medium and those of the extraradical phase of VAM fungus, which normally grows in a

relatively minimal medium. The simplest method of co-cultivating the two organisms (Mosse and Hepper, 1975), where they used a divided plate, where the distal parts of the roots was grown in a mineral medium lacking sugar and vitamins while their proximal part was grown in a complete medium.

Miller-wideman and Watnud (1984) used 0.1 x Murashige and Skoog (MS) medium for inducing initiation of rooting from tomato shoot radicle transition zone. Then VAM fungus *Gigaspora margarita* was established on these roots resulting in colonization of 85 %.

Mugnier and Mosse (1987) studied the establishment and nutrient transfer between the two organisms. They used a divided culture system in which, the proximal ends of roots was grown in a complete medium, where as the root tips in a separate compartment containing water agar and peat. Only root tips were then inoculated with VAM spores and studied for their infection and fungal growth.

A modified White's medium was used by Becard and Fortin (1988) for establishing clonal clover root cultures with *G. mosseae* and with *G. margarita* in transformed root cultures. They continually used the modified White's medium for most of the root organ cultures since it supplements the nutritional requirement compared to MS medium which is considerably rich and must be diluted. They published modified White's medium with appropriate phosphorus and sucrose levels for root organ culture. The reducing levels of these two components was a determining factor in the achievement of colonization. High phosphorus level is

detrimental to VA mycorrihizal establishment and low concentration of sugar promotes fungus establishment in root organ culture. The medium described by Becard and Fortin (1988) was successfully used for dual culturing in the consecutive years (Becard and Piche, 1989a,1989b and 1990). This medium was convenient for dual culture of *G. intraradices* with transformed roots of carrot and tomato. However, non-transformed roots needed regular subculturing on complete White's medium.

Ri T-DNA Transformed roots :

Ri T-DNA transformed roots offer a clean method for growing transformed colonized roots, even though VA mycorrhizal infection in root organ culture was published by Mosse and Hepper (1975). The dual culture with Ri T-DNA transformed carrot roots for 'Independent' growth of VA endophytes was published by Mosse in 1987. Mycorrhizal infection was well established and profuse development of arbuscule like branches were seen on transformed roots with *Glomus intraradices*. The rationale of this approach of root organ culture is discussed.

The early events of VA mycorrhizal formation on Ri T-DNA transformed roots with *Gigaspora margarita* was reported by Becard and Fortin (1988). They studied the initial events of mycorrhiza formation and obtained abundant viable and aseptic spores concentrating much on critical nutritive components of culture

medium like sucrose, sodium and phosphorus for successful establishment of dual culture. Increasing P from 1.08 mg/l to 4.34 mg/l resulted in complete inhibition of infection by *G. margarita* and increasing the sucrose from 1 to 3% dropped infection from 83 to 7%. It came down to 40% when sodium sulphate (453 mg/l) was added to minimal medium. Sporogenesis was regularly observed from the first month to seventh month of dual culture with more than 100 spores produced per plate.

Becard and Piche (1989a) discussed the new aspects of the acquisition of biotrophic status by VA mycorrhizal fungus *G. margarita*. An *in vitro* system of VAM fungus with Ri T-DNA transformed root to investigate the dependency of VAM on its host during mycorrhizal establishment was discussed. The growth of hyphae from germinating spores of *G. margarita* was measured under different combinations, like presence or absence of root etc. They concluded that it is exclusively root dependent for establishment of biotrophy. They also studied the effect of root metabolites on hyphal development from spores of *G. margarita*. Hyphal growth was greatly stimulated by volatile substances and exudates produced by roots. For the first time they demonstrated that carbon dioxide a critical root volatile involved in the enhancement of hyphal growth.

Becard and Piche (1990) reported the physiological factors determining VA mycorrhiza formation in host (carrot) and non-host (sugar beet) Ri T-DNA transformed roots. Formation of infection units and rapid growth of hyphae were seen only in the presence of carrot roots. Hyphal growth was also stimulated by root

exudates of carrot and not by sugar beet. The result suggests that certain factors are essential for mycorrhizal infection, which is lacking in non-host like sugar beet.

An *in vitro* model to identify VAM-plant symbiosis was proposed by Balaji *et al.* (1994). Two nodulating, non-nitrogen fixing (nod $^{+}$, fix $^{-}$) pea mutants on transformed root culture of *A. rhizogenes* were resistant to VAM (*G. margarita*) colonization *in vitro*. Mycelium developed on root surface but failed to colonize. A nodulating (nod $^{+}$) genotype, which was unable to fix nitrogen (fix $^{-}$) in association with *Rhizobium* and a parental line (nod $^{+}$, fix $^{+}$), interacted normally and showed extensive internal colonization. These results confirm that defective nodulation is correlated with defective mycorrhization under axenic conditions.

Nuutila *et al.* (1995) studied the hairy root cultures of strawberry infected with arbuscular mycorrhizal fungus *Glomus fistulosum*. Maintenance of clonal cultures, growth and nutrient uptake were characterized in shake flasks and in a bioreactor. During cultivation vegetative spore formation was observed and hyphae and arbuscules were also observed in stained roots.

A stepwise, procedure was investigated to determine the establishment of *Glomus mosseae, in vitro* culture with Ri T-DNA transformed roots *Daucus carota* (Douds, 1997). Growth of hyphae with germinated spores was much greater in the presence of Tris buffered medium of pH values near neutral (7.2 pH) and purified gelling agent gellon gum used to lower the P concentration of the medium ; with all these factors mycorrhiza were established in 14% of dual cultures.

MATERIAL AND METHODS

III MATERIAL AND METHODS

The transformation of plants involves stable introduction of DNA from desired organism. The major problem in the development of a transformation system is accelerating the provision for inducing *Agrobacterium rhizogenes* to plant cells capable of hairy root induction. The identification of proper host plant for proliferation of hairy root is essential. An effort was made to study the best suitable host media for root organ culture.

The initial experimental studies were conducted to develop suitable protocol for an efficient *in vitro* regeneration of plant host material for *Agrobacterium rhizogenes*. Further, the production of cloned hairy roots on suitable tissue culture media and profuse multiplication of cloned roots was studied. Finally, these Ri T-DNA transformed cloned roots were used axenically for the induction of VA mycorrhizal colonization. The materials and methods for conducting these experiments are as follows.

Selection of explant source and standardisation of media :

Agrobacterium rhizogenes induces hairy roots on dicot plants and the efficiency of this process is more on solanaceous family. Recent studies indicate that the transformation can also be achieved in monocot plants like paddy and

grasses. For selecting an efficient explant for *in vitro* hairy rooting, following host plants were selected.

i) Medicinal plants : (The plants maintained in the Horticulture Department, UAS, GKVK, Bangalore were used for this purpose).

1) Mint (*Mentha piperita*)
2) Mint (*Mentha citrata*)
3) Basil (*Ocimum gratissimum*)
4) Vasaca (*Adathoda vasica*)
5) Coleus (*Coleus aromaticus*)

ii) List of other host plants selected includes,

1) Green gram (*Vigna radiata*)
2) Cowpea (*Vigna unguiculata*)
3) Capsicum (*Capsicum annum*)
4) Carrot (*Daucus carota*)
5) Tomato (*Lycopersicon esculentum*)

Micro-propagation techniques :

From the above listed host plants explants were selected. The part of the organ culture best suited for the purpose was investigated. For this purpose suitable tissue culture media and combination of growth regulators and other aspects were studied.

Composition of tissue culture media :

The tissue culture media used for the experiments were modified Murashige and Skoog medium (1962), Nitch medium (1951), Gamborg B_5 medium (1968), White's and modified White's medium (Becard and Fortin, 1988). The composition of these media and that of minimal medium are given in Table 1 to 4.

Preparation of stock solutions :

The inorganic constituents of each group were weighed separately in a single pan analytical electronic balance, and dissolved individually in double distilled water. The dissolved solution was made up to a known volume in a volumetric flask and stored in stock solution bottles.

The stock solutions of auxins - indole - 3 acetic acid (IAA), naphthalene acetic acid (NAA), indole –3- butyric acid (IBA) were dissolved separately in minimal quantity of redistilled ethyl alcohol and volume was made up with double distilled water.

All the above stock solutions were stored in a refrigerator at 4 to 5^0 C. The temperature of these stock solutions was brought to the ambient temperature before using them for the media preparations.

Table 1 : Composition of modified Murashige and Skoog (MS) medium (1962)

Ingredient Group	Salt concentration in stock solution (mg)	Stock volume made up (ml)	Aliquot taken for 1 l. medium (ml)	Final concentration of salt in 1 l. medium (mg)
INORGANIC SALTS				
I. KNO_3	19000			1900
NH_4NO_3	16500	250	25	1650
$MgSO_4.7H_2O$	3700			370
KH_2PO_4	1700			170
II $CaCl_2.H_2O$	4400	100	10	440
III. $FeSO_4.7H_2O$	278			27.8
Na_2 EDTA.2 H_2O	373	50	5	37.3
IV. $MnSO_4.4H_2O$	223			22.3
$ZnSO_4.7H_2O$	86			8.6
H_3BO_3	62	100	10	6.2
KI	8.3			0.83
$CuSO_4.5H_2O$	0.25			0.025
$NaMOO_4.2H_2O$	2.5			0.25
$CaCl_2.6H_2O$	0.25			0.025
V. VITAMINS				
Glycine	20			2
Myoinositol	1000			100
Thiamine HCl	1			0.1
Pyridoxine HCl	5			0.5
Nicotinic acid	5			0.5
Biotin	0.5			0.05
VI. Sucrose				30000
VII. Agar or				8000
Gelrite				2000
VIII. pH	5.8			

Table 2 : Composition of Nitch medium (1951)

Ingredient Group	Salt concentration in stock solution (mg)	Stock volume made up (ml)	Aliquot taken for 1 l. medium (ml)	Final concentration of salt in 1 l. medium (mg)
INORGANIC SALTS				
I. KNO_3	20000			2000
$MgSO_4.7H_2O$	2500	250	25	250
$NaH_2PO_4.2H_2O$	2500			250
II. $CaCl_2.2H_2O$	250	100	10	25
KCl	15000			1500
III. Ferric citrate	100	50	5	10
IV. $ZnSO_4.7H_2O$	5			0.5
$MnSO_4.4H_2O$	30			3.0
H_3BO_3	5			0.5
$CaSO_4.2H_2O$	2.5	100	10	0.25
$Na_2MOO_4.2H_2O$	2.5			0.25
V. VITAMINS	5			
Nicotinic acid	50	100	10	0.5
Pyridoxine HCL	5			5.0
Ca Pentathenate				0.5
VI. SUCROSE				30000
VII Agar or				8000
Gelrite				2000
VIII. pH	5.8			

Table 3 : Composition of Gamborg -B_5 medium (1968)

Ingredient Group	Salt concentration in stock solution (mg)	Stock volume made up (ml)	Aliquot taken for 1 l. medium (ml)	Final concentration of salt in 1 l. medium (mg)
INORGANIC SALTS				
I. KNO_3	25000			2500
$NH_4 NO_3$	1500	250	25	150
$NaH_2PO_4. 2H_2O$	1500			150
$(NH_4)_2 SO_4$	1340			134
II. $CaCl_2. 2H_2O$	2200	100	10	220
III. NaFeEDTA	280	50	5	28
IV. $MnSO_4.H_2O$	100			10
$ZnSO_4.7 H_2O$	200			20
$H_3 BO_3$	30	100	10	3
KI	7.5			0.75
$CuSO_4. 5 H_2O$	0.25			0.025
$NaMOO_4.2 H_2O$	2.5			0.25
$CaCl_2. 6 H_2O$	2.5			0.25
V. VITAMINS				
Myoinositol	1000			100
Thiamine HCl	100			10
Nicotinic acid	10	100	10	1
Pyridoxine HCl	10			1
VI. Sucrose				30000
VII Agar or				8000
Gelrite				2000
VIII. pH	5.8			
IX.				

Table 4 : Composition of White 's medium, modified White's medium and minimal medium (Becard and Fortin, 1988)

Ingredient Group	White's medium (mg/l)	Modified White's medium (mg/l)	Minimal medium (mg/l)
INORGANIC SALTS			
$MgSO_4.7\ H_2O$	731	731	731
$Na_2SO_4.10\ H_2O$	453	453	-
KNO_3	80	80	80
KCl	65	65	65
$NaH_2PO_4.2H_2O$	-	21.5	-
KH_2PO_4	4.8	-	4.8
$Ca\ (NO_3)_2.4\ H_2O$	288	288	288
NaFeEDTA	8	8	8
KI	0.75	0.75	0.75
$MnCl_2\ .H_2O$	6	6	-
$ZnSO_4.7H_2O$	2.65	2.65	2.65
H_3BO_3	1.5	1.5	1.5
$Cu\ SO_4.5H_2O$	0.13	0.13	0.13
$Na_2MoO_4.2H_2O$	0.0024	0.0024	0.0024
VITAMINS			
Glycine	3	3	3
Thiamine HCl	0.1	0.1	0.1
Pyridoxine HCl	0.1	0.1	0.1
Nicotinic acid	0.5	0.5	0.5
Myo inositol	50	50	50
Sucrose	10000	30000	10000
pH	5.8	5.8	5.8
Agar	10000	10000	10000

Stock solutions of antibiotics used in the study was prepared by dissolving required quantity in sterile double distilled water. Then the solutions were passed though membrane filter (0.22 µm pore size) and kept in a sterile reagent containers at 4^0 C until use. Stock solutions of antibiotics stock solutions were thawed before use and the required quantity was mixed with media before plating.

Preparation of culture medium :

The required quantity of sucrose was weighed and dissolved in double distilled water. Different volumes of the stock solutions as mentioned in the media (Tables 1 to 4) were pipetted out along with different growth regulators and chemical based on the requirements of the experiment. The volume was made up using a volumetric flask. The pH of the medium was adjusted to 5.8, using 0.1 N hydrochloric acid or 0.1 N NaOH, with the help of a digital pH meter. A known quantity of agar (1.2%) was used and the culture media were sterilized in an autoclave at a temperature of 121^0 C and a pressure of 1 kg/sq. cm. for 20 minutes and stored in a dust proof chamber.

***In vitro* seed culture and sterilization of explants :**

Pretreatment of seeds :

Seeds of cowpea, green gram and capsicum were used for *in vitro* seed culture. The seeds were sterilized by using 0.1% $HgCl_2$ and sodium hypochlorite (0.5% chlorine) and washed with sterilized water for 4 to 5 times. Seeds were then

aseptically transferred on to a sterilized filter paper in a petridish and kept for germination. After 10 to 15 days of germination, the shoot and root portions were excised and used as explants on MS basal medium.

Selection of plant part as explant for root induction :

i) Leaf disc method :

Leaf disc method of *Agrobacterium* mediated transformation was followed in the current study. Leaf pieces of size approximately 1.0 cm^2 were cut with leaf punch. Leaf pieces were surface sterilized with sodium hypochlorite (0.5% chlorine) for 5 minutes and later rinsed with sterile water for 4-5 times. Leaves of cowpea, green gram, capsicum, tomato and medicinal plant leaves were subjected to this test.

ii) Plant stem as explants :

Tomato (*Lycopercicum esculantum* var. Pusa Ruby), cowpea (*Vigna unguiculata* var. C-152) and green gram (Local variety) plants were grown under green house condition. Parts of the plant were collected at different intervals from 15 to 60 days after germination to assess the age of the material for better induction of rooting. Plant shoot organs such as matured stem, leaf petioles were used as explants.

Sterilization of explants :

Explants of cowpea, green gram and capsicum and medicinal plants :

Leaf discs of 1 cm^2 used were sterilized by 2% chloramine T and sodium hypochlorite (0.5%) for 3-5 minutes based on age and thickness of the leaf material.

Leaf petioles and stem portion when used as explants, were collected and washed thoroughly in running water. Then few drops of Tween-20 was added and washed again. The plant materials were then cut into 7 to 8 cm bits. These larger bits were then surface sterilized by using hypochlorite for 5-7 minutes based on the age of the material. They were rinsed 4 to 5 times thoroughly with sterile water. The materials were then used for co-culturing.

Tomato explant :

The preliminary experiment suggested that tomato stem serves as the best source of explant material for transformation studies. The standard protocol developed for producing root organ culture from tomato stem is given below :

Protocol for sterilizing tomato stem explants :

1) Matured stem portions as explants (2 months old plants) were selected and cut into 7 to8 cm bits.
2) The stem pieces were transferred to 500 ml conical flask and washed thoroughly with tap water.

3) Further washing was done with 100 to 200 ml of double distilled water to which 0.5 ml of Tween-20 was added.
4) The stem pieces were again washed with running water.
5) Then the stem bits were transferred to sodium hypochloite (2% available chlorine) for 5 to 7 minutes.
6) After decanting sodium hypochlorite, the stem bits were rinsed with sterile water for 4 to 5 times.
7) The sterile explants were then transferred on to sterile filter paper.
8) Using sterile knife or scalpel both the ends were trimmed and then the explants were cut into 2 to 3 cm bits. These bits were co-cultured with *Agrobacterium rhizogenes.*

Steps 5 to 8 were carried out in a laminar flow.

Effect of growth regulators along with different levels of sucrose on the initiation of rooting :

Basal MS medium with four levels of sucrose (1.5, 2.5, 3.5 and 4.5%) along with different concentrations of auxins (IAA, IBA and NAA) at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l were used. Five pieces of sterilized explants (as explained above) were placed in a petridish containing the medium and incubated at 25^0 C. Observations on callus formation and root initiation were made after 8^{th} and 12^{th} day of incubation. Five replicate plates were maintained for each treatment.

Selection of culture medium for different strains of *Agrobacterium rhizogenes*, used in the study :

Wild strains of *Agrobacterium rhizogenes*, are known to induce hairy root in most of the crop plants. In order to ascertain the process of transformation through *Agrobacterium*, 10 wild strains were collected from different sources as given below:

Strains	Source	Medium on which maintained
1) MTCC – 532	CTCRI, Trivandram	YEA
2) ATCC – 3126 C	-do-	-do-
3) ATCC – 15834	NCL, Pune	NA
4) A_4	-do-	-do
5) MTCC – 533	CIMAP, Lucknow	YEMA
6) Strain – 8196	-do-	-do-
7) LBA – 9402	University of Washington, USA	YEMA
8) A_4	-do-	-do-
9) ATCC – 15834	Leiden University, Netherlands	YEMA
10) A_4	Madurai Kamaraj University, Madurai	YEA

Different laboratories from where the strains were obtained had specified different culture media for growing *A. rhizogenes* strains. Hence, it was decided to standardise a common suitable medium for the growth of most of the strains of

A. rhizogenes. Yeast extract mannitol broth, yeast extract broth, nutrient broth and modified yeast extract broth were used.

1) Yeast extract mannitol broth (YEMB) (Fred *et al.*, 1932)

Mannitol	10.0 g
Yeast extract	1.0 g
K_2HPO_4	0.5 g
$CaCl_2$	0.2 g
NaCl	0.2 g
$MgSO_4$. 7 H_2O	0.2 g
$FeCl_2$	0.01 g
Water	1000 ml
pH	7.0

2) Yeast extract broth (YEB)

Mannitol	10.0 g
Mg SO_4. 7 H_2O	2.0 g
K_2HPO_4	0.5 g
NaCl	1.0 g
Water	1000 ml
pH	7.00

3) Modified yeast extract broth (MYEB)

Mannitol	10.0 g
Yeast extract	0.5 g
K_2HPO_4	0.2 g
$MgSO_4.7\ H_2O$	0.2 g
NaCl	0.1 g
Water	1000 ml
pH	6.8

4) Nutrient broth (NB)

Peptone	3.0 g
Beef extract	5.0 g
NaCl	5.0 g
Glucose	5.0 g
Water	1000 ml
pH	7.0

The growth was scored based on density of growth.

***Agrobacterium rhizogenes* transformation :**

The co-culturing of explants and bacterial strains was established for hairy rooting by the following procedure :

Experimental details :

Preparation *Agrobacterium rhizogenes* inoculum :

Agrobacterium rhizogenes strains from different sources were selected based on their growth and other characters. The selected strains were streaked on respective culture media and were grown for 48 hrs at 28^0 C. The growth of *Agrobacterium* was monitored by measuring the OD at 660 nm. The culture having a OD of 0.8 to 1.0 was selected for infecting the explants.

Preparation of tissue culture media and antibiotics :

MS basal medium was used for the initial establishment of explants. The constituents of MS Basal medium is given in Table 1. A stock solution of 250 mg/ml of antibiotic cefetoxime was freshly prepared and filter sterilized (0.22 µm membrane and stored in a sterile container.)

Co-Culturing :

The above prepared sterilized explant bits were carefully transferred to sterile petriplate that contained *Agrobacterium rhizogenes* culture. The explants were treated for 20-30 minutes and then blotted on sterile filter paper to remove excess culture adhering to the bits. Then they were transferred to MS Basal medium without antibiotics and were incubated for 48 hrs at 25^0 C to allow for infection process.

After 24 hours if any over growth of bacteria was seen, the explants were transferred to selection medium (MS medium + 500 mg/l of cefetoxime). Then it was observed for callus and root induction. A descriptive protocol is given below.

Protocol for co-culturing with *Agrobacterium rhizogenes* :

1) Sterilized explants were aseptically transferred on sterile filter paper.
2) With sterile blade the edges were trimmed to remove dead cells.
3) The explants were cut into small pieces (2 to 3 cm. bits).
4) The explants were transferred to petriplate containing 48 hrs old culture of *A. rhizogenes* and were treated for 1 to 2 hrs.
5) Again the pieces were transferred on sterile filter paper to remove excess of culture adhering to explants.
6) Then they were transferred to MS basal medium with 30% sucrose (about 4 to 6 bits/plate).
7) The plates were incubated at 25^0 C for 48 hours in darkness.

Establishment and maintenance of cloned roots :

After sufficient incubation of explants on MS basal medium, the callus induction takes place and may lead to rooting. To establish this transformation process and to maintain the cloned roots a separate protocol was standardised which is given below :

Protocol for establishment of transformation :

1) After the explants were transferred on MS basal medium, the growth of bacteria was looked for.
2) If growth appeared, then immediately it was transferred to petriplate with MS + 500 mg/l antibiotics (Ab).
3) After 24 hours, 2nd transfer was done on MS + 500 mg/l Ab.
4) After 24 hours 3rd transfer was done on MS + 500 mg/l Ab.
5) This process was continued as follows

   4th transfer on MS + 250 mg/l Ab.

   5th transfer on ½ MS + 125 mg/l Ab.

   6th transfer on ½ MS + 100 mg/l Ab.

   7th transfer on ½ MS + 50 mg/l Ab.

6. Finally it was transferred on plain ½ MS medium and during 4th and 5th transfer, search was made for callus induction and rooting.

Maintenance of Transformed root :

If the cloned roots are produced on 4th or 5th transfer, the antibiotic treatment was reduced and brought to only ½ MS plain medium. Then final maintenance was on minimal medium without antibiotics. These were maintained generally in dark at room temperature . They were transferred to fresh medium at 4 to 6 weeks intervals.

Effect of incubation temperature on growth of cloned roots :

Since most of the tissue culture works was done at low temperature, the experiment was conducted to know the effect of low temperature (25^0 C) and (30^0 C) on cloned roots. To study the effect of different temperatures on cloned roots the following experiment was conducted.

Experimental Set up :

Cloned roots growing on minimal medium (MM) were excised into 10 to 15 mm bits on MM. About 2 bits were maintained in each plate keeping 10 replicate plates for each treatment. They were incubated at different growth temperatures of 25 and 30^0 C. Increase in root length was recorded at timely interval of 4, 8, 12, 16 and 20 days after incubation. The results were tabulated and statistically analysed.

Opine test for the confirmation of transformation by *A. rhizogenes* :

A. rhizogenes on transfer of T-DNA in the host induces the production of certain specific compounds that have been biologically characterized as opines. These substances are agropine, mannopine, mannopinic acid and agropininc acid. According to opine content of hairy root tissues, two types of *Agrobacterium rhizogenes* strains have been identified – agropine type and mannopine type strains.

The procedure followed for the identification of opine was that described by Petit *et al.* (1983).

High voltage paper electrophoresis for identification of opines :
Preparation of transformed root extract :

Hairy root segments were placed in preweighed Eppendroff tubes. The segments of (0.5 to 10 mg) were extracted twice with 40 µl of boiling double distilled water in 10 minutes. The combined extracts were evaporated to dryness in a vaccum centrifuge equiped with a cold trap. The dried extracts were dissolved in double distilled water (2 µl water per mg of sample).

High voltage paper electrophoresis :

Extracts of 2 to10 µl were spotted on 3 mm Whatman filter paper and subject to high voltage paper electrophoresis (ca.100 v/cm. for 8-15 min.) (Gilson high electrophoresis model D). Developing was done using formic acid/acetic acid/distilled water (30/60/910, v/v/v) adjusted to pH 9.8 with concentrated sodium hydroxide. After drying in a current of hot air, the electrophoretograms were stained with dilute silver nitrate as described by Trevelyan *et al.*, 1950 and washed thoroughly for several hours with running water. Standard agropine and mannopine at the concentration of 5 µg/l were also spotted

along with the sample. The darkly stained spots were correlated for the standard opine.

Establishment of VAM on cloned roots :

For establishment of VA mycorrhiza on cloned roots, the VAM-spores were isolated, surface sterilized and used for inoculation (3.13 and 3.14 of the text).

VA mycorrhizal colonization in transformed roots :

From a single transformed root 10 to 20 mm root tips were excised from the clonal culture and transferred on MM medium for 10 days at 25^0 C. A single spore was placed near the root 1.0 to 1.5 cm away from the root tip. Then the petriplates were vertically incubated in such a way that germ tube would grow upward and come in contact with the roots.

Cotton tweets were sterilized separately and a piece of it was kept at the lower portion of the petriplate in order to absorb excess moisture in the plate. The development of extramatrical phase of the VAM fungus was observed using a binocular microscope. Root samples were taken after 8 to 10 days of germination of spore and stained with trypan blue as described by Phillips and Hayman (1970) for observing mycorrhizal colonization.

Effect of temperature on the growth and sporulation of VAM in transformed roots :

In order to assess the effect of temperature on growth and sporulation of VAM fungi in transformed roots the following experiment was set up.

The VAM fungal spores used for the study includes :

1. *Glomus fasciculatum*
2. *Glomus mosseae*
3. *Gigaspora margarita*
4. Isolate –1, *Glomus geosporum*.

The plates were prepared as explained earlier and were incubated at two different temperatures viz., 25 and 30^0 C with 20 replications for each sample. The plates were observed at every 4 days interval under stereomicroscope. The percentage mycorrhizal colonization was carried out after 30 to 35 days of incubation. The percentage colonization of these spores were compiled against the incubation temperature and were statistically analysed.

VAM spore isolation :

The VA mycorrhizal cultures *Glomus fasciculatum*, *Glomus mosseae* and *Gigaspora margarita* required for the study were obtained from the Department of Agricultural microbiology, University of Agricultural Sciences. GKVK, Bangalore. The cultures were maintained either in 1:1 sand : soil or 1:1 soilrite : perlite mixture using *Panicum maximum* as the host. The other culture (Isolate – 1) used in the

study was from a mine spoil rich in VAM spores. The isolated spores were then used for identification.

VA mycorrhizal spore isolation :

The VAM spores were isolated by wet sieving and decantation method of Gerdemann and Nicolson (1963). A known quantity of VAM inoculum was suspended in 1000 ml. of water and stirred thoroughly until if formed a uniform suspension. Then the suspension was passed through a series of sieves of sizes 1 mm, 450, 250, 105 and 45 μm, arranged one below the other in the same order as mentioned above. The majority of the spores were collected from both 105 μm and 45 μm sieves. The spore suspensions were mixed and used for further isolation.

Concentration and purification of isolated VAM spores by density gradient centrifugation method :

After isolating VAM spores by wet sieving and decantation, further purification of spores by picking them using capillary tube under stereomicroscope is more cumbersome and time consuming. Hence, further purification of spores is usually done by sucrose gradient method suggested by Furlan *et al.* (1980). This method has some disadvantages as sucrose if not washed properly leads to contamination. Further, mixing of the gradients also occurred easily. Hence, a modified density gradient method using glycerol in place of sucrose was attempted. This method is outlined below :

1) The spores were collected by wet sieving and decantation method (described earlier).
2) Spore suspension was made up to 30 ml in 50 ml centrifuge tube.
3) Centrifugation was done at 2500 rpm for 5 minutes.
4) The spore pellet was collected in distilled water.
5) Gradients were made by glycerol 60% and 30% (by adding few drops of 2% safranine solution to 60% glycerol to get pink colour).
6) 10 ml of 60% glycerol was added followed by 10 ml of 30% glycerol in 50 ml centrifuge tube.
7) 10 ml of spore pellet was added slowly on 30% glycerol without disturbing the gradients.
8) Centrifugation was done at 250 rpm for 5 to 7 minutes.
9) The spores collected between the gradients 60 and 30 were separated with a syringe (60% gradient has pink colour).
10) The spore suspension was washed in distilled water for 2 to 3 times and number of spores per quantity of inoculum taken was counted.

The spores collected from the above method was compared with the spores purified by the method explained by Furlan *et al.* (1980) using sucrose gradients. The number of spores recovered by these methods were compared and expressed in percentage.

VAM spore sterilization :

For axenic culturing of VAM fungi with cloned roots, a high quality surface sterilized VAM-spores are essential. Sterility of spores prevents other natural contaminants especially in tissue culture media.

The spores were surface sterilized by the method described by Mertz *et al.* (1979) with a slight modification. A special funnel apparatus was designed for effective and accurate sterilization.

All sterilization processes were carried out in laminar flow cabinet. Spores were washed in 0.05 % (V/V) Tween-20 solution with 2 % chloramine T (W/V) taken in a glass vial. A low pressure was created by drawing air out from the vial by syringe. This creates a light vacuum in order to remove liquefied gases and air droplets adhering over the spore surface. There after spore suspension was transferred to sterilized filter paper (Whatman No.1) placed in a funnel, the tube of which was fitted with a tap. Here the spores were thoroughly washed with sterile distilled water in order to remove Tween-20 and then treated again with chloramine-T for 20 minutes.

After thorough washing with sterile water, the spores were subjected to antibiotic solutions I and II . The spores were treated with antibiotic solution I (gentamycin 100 mg/l and streptomycin 200 mg/l) for 2 minutes and rinsed with

several changes of sterile water. The spores were then treated with antibiotic solution II (cephalexin 250 mg/l) for 5 minutes. The antibiotic solution was completely drained and spores washed thoroughly with sterile water.

At this stage the spores could be stored at 4^0C for 2 to 3 months on the same filter paper. Spores could also be used immediately following 4 to 5 washings in sterile water. Stored spores has to be treated with chloramine – T and antibiotics I (as described earlier) before use. Only white to creamy spores were selected and picked up with a fine sterilized injection needle and were used for the study.

Effect of varying concentration of crude cellulase in the medium on VAM spore germination :

The experiment was conducted to observe the effect of varying concentration of crude cellulase on spore germination and germ tube development. Water agar was supplemented with different concentrations enzymes viz., 80, 160, 240, 320 and 400 µg/l. There were six replications with 25 spores per plate. The germination and germ tube growth were recorded at an interval of every four days up to the end of 3rd week. VAM spores used were viz., *G. fasciculatum*, *G. mosseae*, *Gigaspora margarita*, and isolate – *Glomus geosporum*.

Influence of bacterial cell free extracts on germination of VAM spores :

The present experiment was designed to select the best level of bacterial cell free extracts enhancing the VAM spore germination. The experimental details are given below :

Bacterial cultures (cell free extracts) :

Bacillus megatherium

Bacillus coagulans

Azospirillum brasilense

Azotobacter chroococcum

Rhizobium sp.

Cultures of 45 to 60 days old cell free extracts were collected and filter sterilized (0.22 µm) and were used at three levels of 2.5, 5.0 and 10.0 ml/100 ml of plain agar. There were six replications with 25 spores per plate. Spore germination was recorded at every fourth day interval up to 3 weeks.

Influence of fungal cell free extracts on germination of VAM spores :

The following beneficial fungal cultures were used for the study,

Trichoderma harzianum

Aspergillus awamori

Aspergillus sp. (LC_4)

Trichoderma sp. (LC_5)

Phenocheate chrysosporium

Cultures of 45 to 60 days old cell free extracts were used after removing fungal mantle from the culture flasks. Cell free extracts were filter sterilized using 0.22 µm size membrane filter and three levels of cell free extracts of 2.5, 5.0 and 10.0 ml/100 ml of plain agar were used. Six replications with 25 spores per plate were maintained and spore germination was recorded at every fourth day interval up to 3 weeks.

Identification of VAM spore isolated from mine spoil :

The isolate - 1 was used in the study was isolated from a mine spoil where only grasses were growing. The score population was quite high in the spoil. After wet sieving individual spores were picked up by using pasture pipettes under stereomicroscope.

A similar morphological type of spores was predominantly present. These spores were mounted on a glass slide with a drop of lactoglycerol. The spore observations such as spore dimensions, spore colour, shape, surface structures, type of hyphal attachment and general nature of spore contents were studied. Spore dimensions were done using standarised ocular micrometer. Mean values of spore diameter and hyphal diameter were recorded. A diagnostic slide containing both broken and unbroken spores were prepared and identification of spore was done referring to the manual for the identification of VA mycorrhizal fungi (Schenck and Perez, 1988).

Mycorrhizal colonization of roots :

The root samples from dual culture plates were collected and preserved in FAA solution (Formalin – acetic acid – alcohol : 90 :5:5).

The FAA preserved roots were washed with water and stained by the method proposed by Phillips and Hayman (1970). The root bits (10 cm) were placed in glass vials having 10% KOH and simmered on water bath (90^0C) for 30 minutes. The KOH was decanted and washed with tap water. The roots were bleached with alkaline H_2O_2 (3ml NH_4 OH + 30 ml of 10 % H_2O_2 + 567 ml water) for 10 to 20 minutes. Then H_2O_2 was removed by rinsing with water and the roots neutralized with 1% HCl for 3 to 5 minutes. Staining was done by simmeing the roots in 0.05% Trypan blue in lactoglycerol (lactic acid 400 ml: glycerol 500 ml : water 100 ml) for 10 minutes. The excess stain was removed and root bits placed in lactoglycerol for observation.

Gridline intersect method :

The stained roots were assessed for VAM fungal colonization by examining for the presence of vesicles, arbuscules and hyphae in the root system. The percentage colonization was determined following the gridline intersect method proposed by Giovannetti and Mosse (1980).

The roots were spread evenly on 10 cm x 10 cm glass plates having 1 cm square gridlines at the other side. The roots were scored as either positive (+) or negative (-) for mycorrhizal colonization at each point of vertical and horizontal gridline intersects. The percentage of mycorrhizal colonization was calculated as follows:

$$\text{Percent VA mycorrhizal colonization} = \frac{\text{Total number of intersection positive for colonization}}{\text{Total number of intersection between roots and gridlines}} \times 100$$

EXPERIMENTAL RESULTS

IV EXPERIMENTAL RESULTS

Selection of suitable culture media for *Agrobacterium rhizogenes* :

Different laboratories which supplied the strains of *A. rhizogenes* had grown them in different media as detailed earlier under material and methods. In order to select one medium in which all the strains can grow, they were grown on four different media viz., yeast extract mannitol broth (YEMB), yeast extract broth (YEB), modified yeast extract broth (MYEB) and nutrient broth (NB).

The results indicated that among four media tested, MYEB was the best in promoting growth of *Agrobacterim rhizogenes* strains (Table 5 and Fig.1). Strains A_4, ATCC - 15834 and LBA - 9402 showed good growth in MYEB followed by YMB a general medium commonly used for growing *A. rhizogenes*.. All the other strains also showed a better performance except strains ATCC – 8196 and MTCC – 533. Very poor growth was seen in other culture media like YMB and NB. Based on these growth results, it was decided to use modified yeast extract broth for routine culturing of *A. rhizogenes*.

Selection of host explants for the induction of hairy root :

A preliminary incubation study was undertaken to select the best strain among *A. rhizogenes* strains. It was shown that three of the ten strains collected for the work were best suited and so, these strains viz., LBA – 9402, ATCC – 15834

Table 5 : Selection of a single culture medium for growing different strains of *Agrobacterium rhizogenes*

Agrobacterium rhizogenes strains	Growth of *A. rhizogenes*			
	Culture media			
	YMB	YEB	Modified YEB	NB
A) ATCC – 532	0.0(0.71)	1.0(1.22)	2.0(1.58)	1.0(1.22)
B) ATCC –15834	0.0(0.71)	1.0(1.22)	1.0(1.22)	0.0(0.71)
C) ATCC – 5834	0.0(0.71)	2.0(1.58)	2.0(1.58)	0.0(0.71)
D) A_4	0.0(0.71)	2.0(1.58)	2.0(1.58)	0.0(0.71)
E) S. No. 8196	1.0(1.22)	0.0(0.71)	0.0(0.71)	0.0(0.71)
F) MTCC – 533	1.0(1.22)	0.0(0.71)	0.0(0.71)	0.0(0.71)
G) A_4	2.0(1.58)	1.0(1.22)	3.0(1.87)	2.0(1.58)
H) ATCC – 15834	1.0(1.22)	1.0(1.22)	2.0(1.58)	0.0(0.71)
I) A_4	2.0(1.58)	1.0(1.22)	3.0(1.87)	0.0(0.71)
J) LBA – 9402	2.0(1.58)	1.0(1.22)	3.0(1.87)	0.0(0.71)

	SEM ±	CD (P=0.05)
Media	0.10	0.30
Strains	0.16	NS

* Statistical analysis are made on transformed values

0.0 – No growth 1.0 – Less growth 2.0 – Good growth 3.0 – Excellent growth

* Values in parenthesis are transformed values.

YMB = Yeast extract mannitol broth
YEB = Yeast extract broth
NB = Nutrient broth

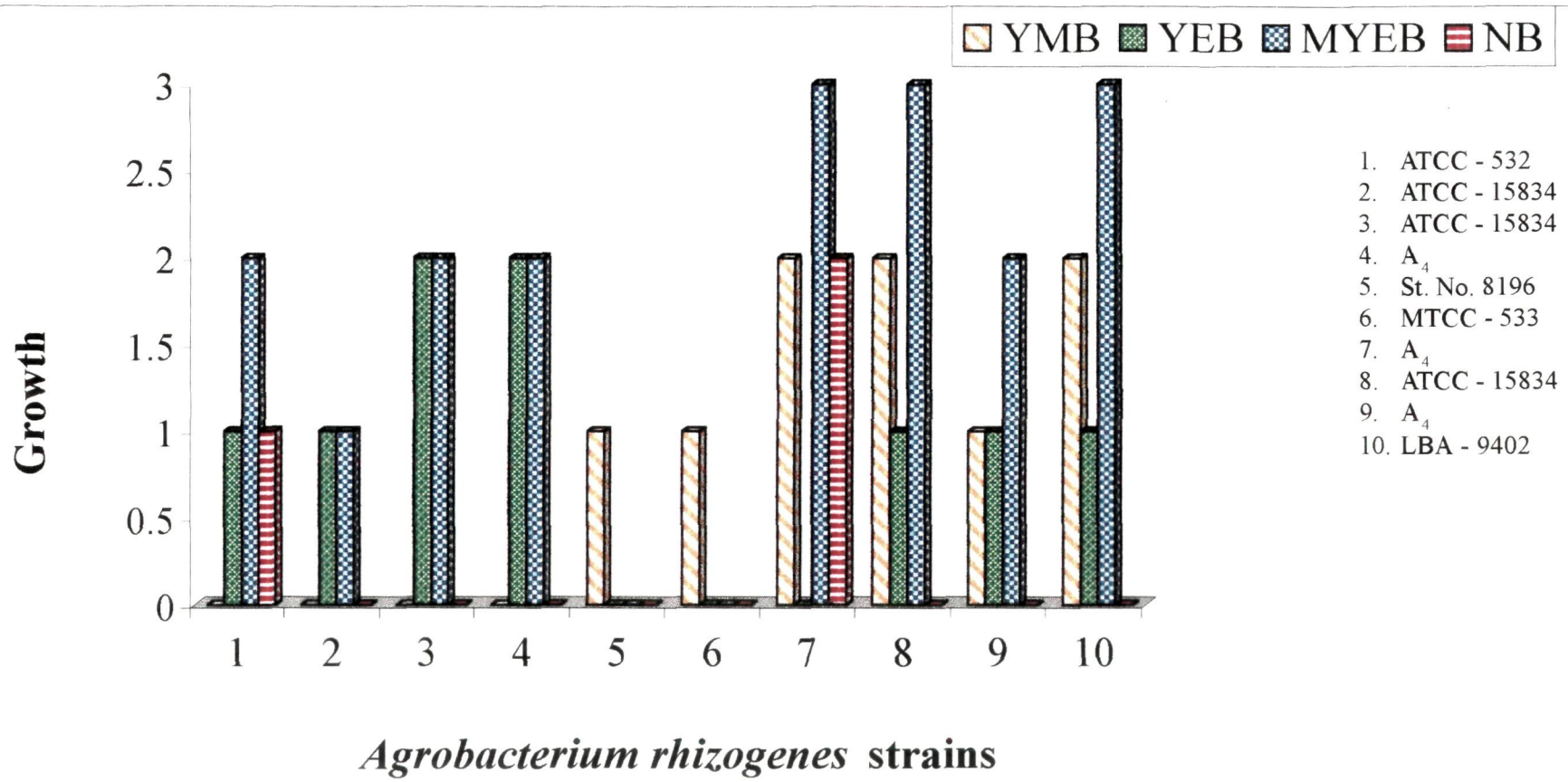


Fig. 1: Selection of single culture medium for growing different strains of *Agrobacterium rhizogenes*

and A_4 strains were selected for future work. LBA – 9404 was the highest inducer of hairy root followed by A_4 and ATCC – 15834. The remaining strains had taken longer time with lesser per cent of root induction.

A. rhizogenes forms hairy roots in most of the dicot plants and especially on solanaceous plants. In the present experiment, combination of ten host explants with five tissue culture media were carried out with *A. rhizogenes* strain LBA – 9402 and the percentage of callus induction in these explants was observed (Table 6 and Fig.2). The method of co-culturing the explants as explained under materials and methods was followed and is shown in Plate 1. Plants organs such as leaf, leaf petiole and stem were used as explants.

Among the medicinal plants mentha (*Mentha piperita*) responded well by co-culturing with *A. rhizogenes* on MS basal medium. The percentage of callus induction was highest (20%) in *Mentha piperita* while *Mentha citrata* showed 16 per cent of root induction on MS medium. Other medicinal plants did not grow well and no callus induction was seen in *Adhathoda*, whereas *Coleus* (12%) and *Ocimum* (4%) responded less. Among the tissue culture media used most of the above percentage of root inductions were seen only on MS basal medium followed by modified White's medium (4-8%) and B_5 medium (0-4%). Nitch medium was not suitable for the micropropagation of medicinal plants.

Table 6 : Screening of different host explants for callus induction by *Agrobacterium rhizogenes* (Strain LBA - 9402) on different tissue culture media

Host explants	Callus induction (%)					
	Tissue culture media					
	MS	WM	MWM	B_5	NM	MEAN
A) *Mentha piperita* (Mint)	20.00 (1.16)	0.00 (0.71)	8.00 (0.91)	4.00 (0.81)	0.00 (0.71)	6.40
B) *Mentha citrata* (Mint)	16.00 (1.11)	0.00 (0.71)	8.00 (0.91)	4.00 (0.81)	0.00 (0.71)	5.60
C) *Ocimum gratissimum* (Basil)	4.00 (0.81)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	1.60
D) *Adhatoda vasica* (Vasaca)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.80
E) *Coleus aromaticus* (Coleus)	12.00 (1.01)	0.00 (0.71)	0.00 (0.71)	20.00 (1.16)	0.00 (0.71)	6.40
F) *Vigna unguiculata* (Cowpea)	36.00 (1.50)	8.00 (0.91)	16.00 (1.11)	28.00 (1.33)	24.00 (1.26)	22.40
G) *Vigna radiata* (Green gram)	28.00 (1.33)	4.00 (0.81)	12.00 (1.01)	0.00 (0.71)	0.00 (0.71)	8.80
H) *Capsicum annum*) (Capsicum)	16.00 (1.11)	4.00 (0.81)	12.00 (1.01)	0.00 (0.71)	0.00 (0.71)	6.40
I) *Daucus carota* (Carrot)	44.00 (1.63)	8.00 (0.91)	32.00 (1.43)	4.00 (0.81)	0.00 (0.71)	17.60
J) *Lycopersicon esculentum* (Tomato)	64.00 (1.84)	16.00 (1.11)	26.00 (1.19)	4.00 (0.81)	16.00 (1.11)	25.20
Mean	24.00	4.00	11.80	6.80	4.00	

	SEM ±	CD (P=0.05)
Host plants	0.04	0.13
Media	0.03	0.09
Interaction	0.10	0.30
CV %	26.10	

* Values in parenthesis are transformed values.

MS - Murashige and Skoog medium
WM - White's medium
MWM - Modified White's medium
B_5 - Gamborg B_5 medium
NM - Nitch medium

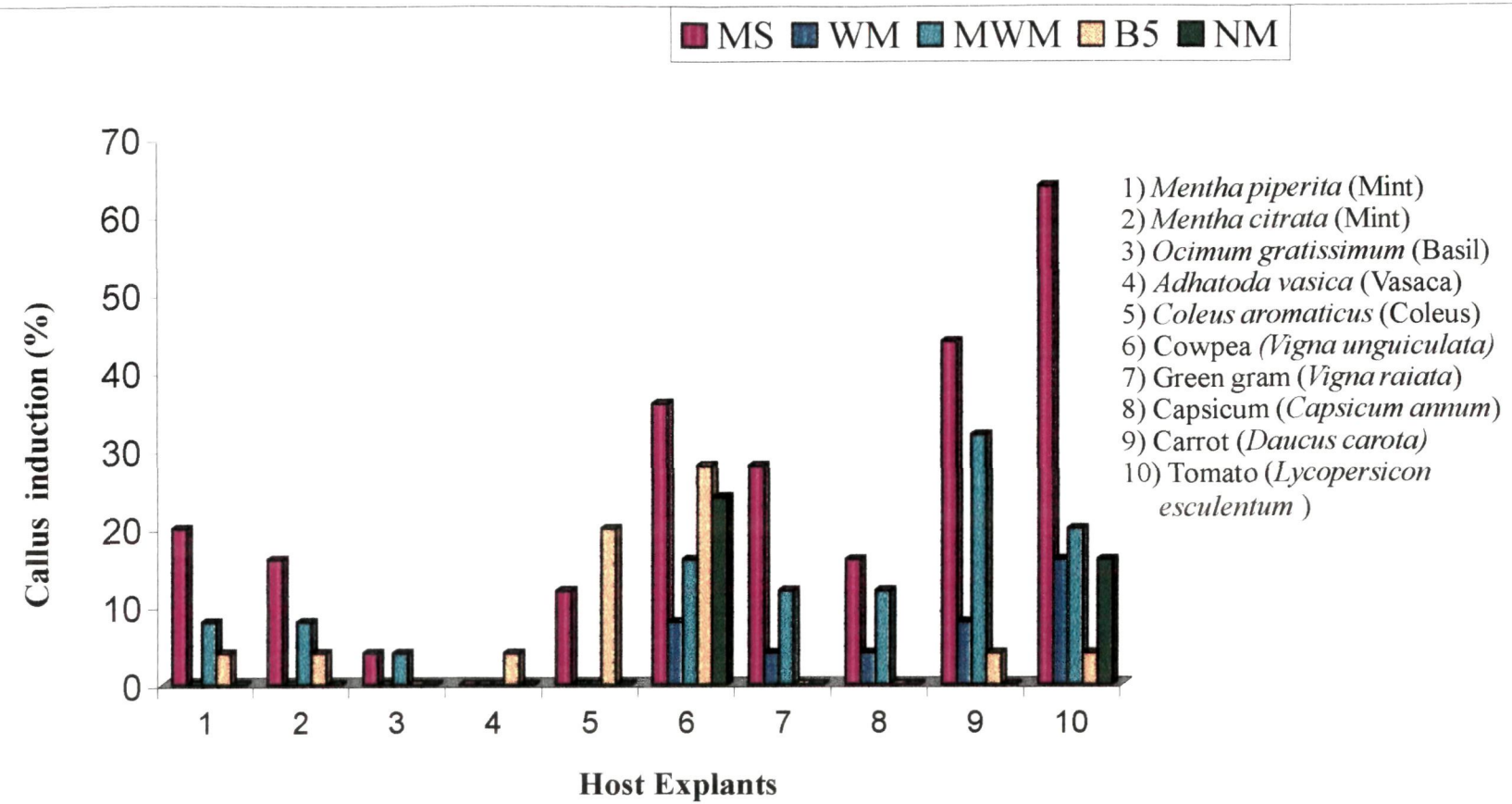
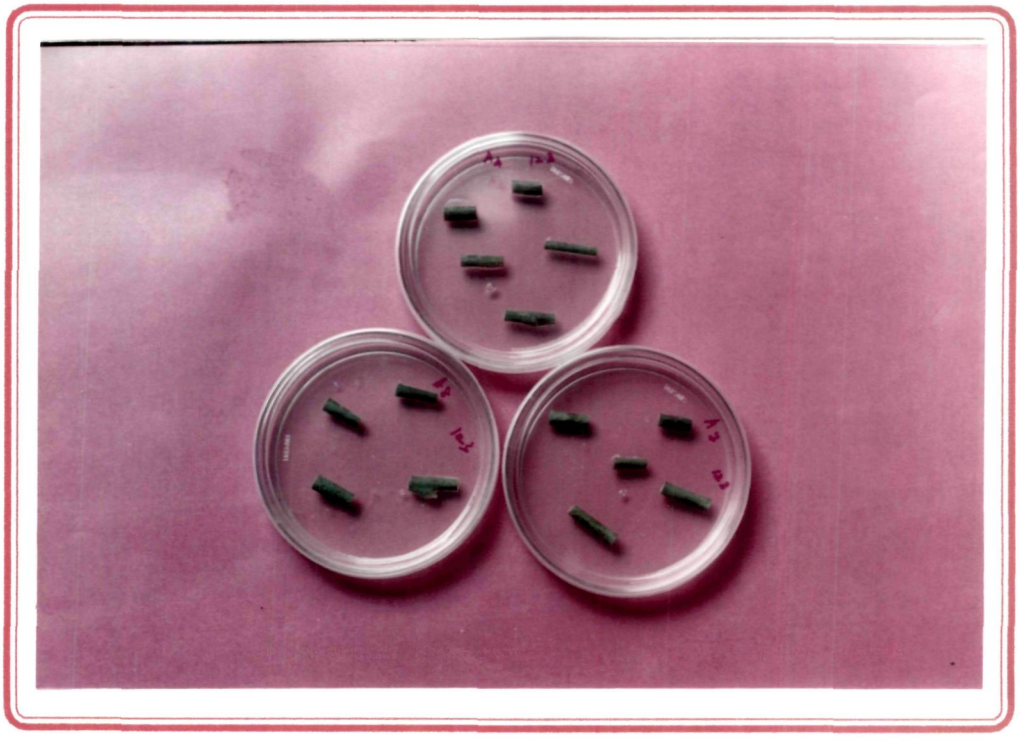


Fig. 2 : Screening of different host explants for callus induction by *Agrobacterium rhizogenes* (Strain LBA - 9402) on ifferent tissue culture media

A



B

PLATE 1: Root induction of different explants with Agrobacterium rhizogenes (LBA-9402)

A. Leaf and petioles of cowpea.
B. Stem explants of green gram.

Crop plants performed better and then the medicinal plants and root formation was quicker. The most suitable plant for the study was tomato. Co-culturing of tomato explants is shown in Plate 2. Root induction in tomato was 64 per cent while in carrot it was 44 per cent, in cowpea it was 36 per cent and in green gram it was 28 per cent. On MS basal medium, callus formation was seen on the 4^{th} day of incubation and it was distinct on the 6^{th} day (Plate 3). Initial stages and typical hairy rooting were also seen from the infected regions of the stem explants (Plate 4).

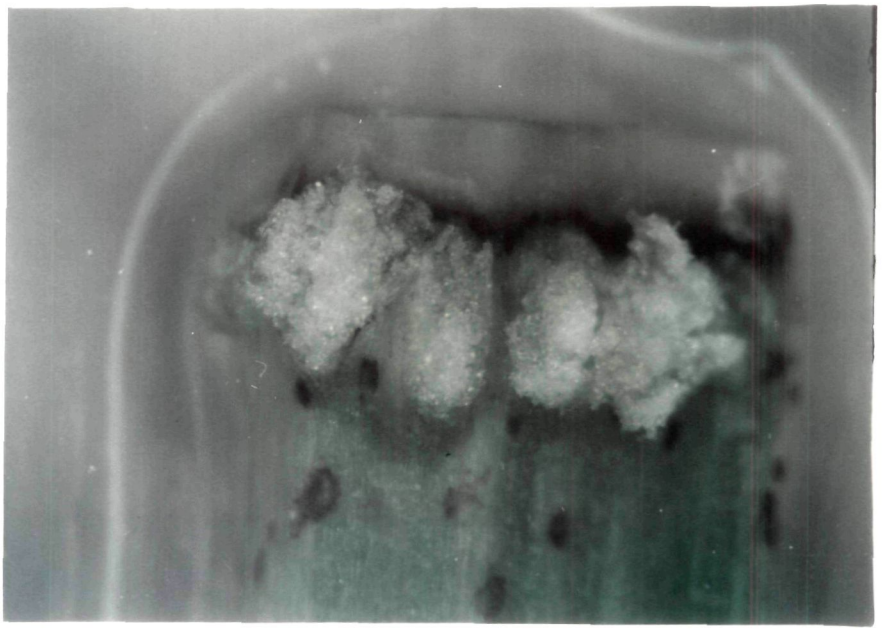
All the plant explants grew well on MS basal medium followed by MWM. It was interesting to observe that cowpea showed root induction on B_5 medium (28%) and on Nitch medium (24%). It had overall induction mean of 22.40 per cent as compared to other crops on all other tissue culture media. MS basal medium also favoured root induction in other plants such as cowpea, capsicum, carrot (root discs) and green gram. On modified White's medium the rate of initiation was 12 – 32 per cent, on White's medium it was 4 – 16 per cent, followed by B_5 medium and the least being in Nitch medium. The statistical analysis of these observations with plants and media and their interaction significantly emphasises that the best medium suited for the work is MS basal medium with a mean root induction of 24 per cent (Table 6). The results also confirmed that the best suited host for inducing hairy roots using *A. rhizogenes* strain LBA – 9402 is tomato.

A

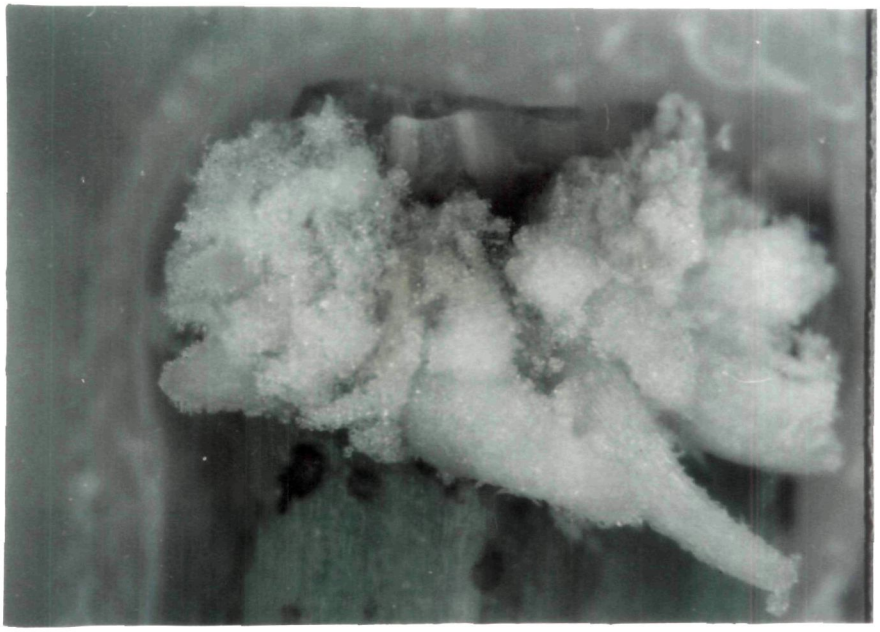
B

PLATE 2: Root induction of tomato stem explants with *Agrobacterium rhizogenes*

A. Strain LBA-9402.
B. Strain A_4



A



B

PLATE 3: Callus formation from tomato stem on MS basal medium on 4th (A) and 6th day (B)

A

B

PLATE 4: Hairy rooting in tomato stem induced by *Agrobacterium rhizogenes* (LBA-9402)

A. Root intiation
B. Typical hairy root formation

A similar experiment was conducted to select the best host explants with *A. rhizogenes* strain ATCC – 15834. Highest root induction was seen with tomato (48%) and cowpea (36%) on MS basal medium (Table 7 and Fig.3). Root induction in the other plant explants were between 12 – 24 per cent. Among the medicinal plants *Coleus* responded well to *A. rhizogenes* strain ATCC – 15834. A similar trend was also seen when *A. rhizogenes* strain LBA – 9402 was used. MS basal medium was the best for root induction with 30 per cent followed by modified White's medium (0 – 24%), White's medium (0 – 8%), B_5 medium (0 – 16%) and least being in Nitch medium (0-4%). Cowpea also showed better root initiation with this particular strain on Nitch medium (20%).

A selective and comparative hairy root induction was finally done with *A. rhizogenes* – A_4 strain (Table 8 and Fig.4). A highly significant result was obtained with A_4 strain with root induction of 48 per cent in tomato, 32 per cent in carrot and 24 per cent in cowpea. The comparative result of root induction by three different strains of *A. rhizogenes* suggests that the best suited host plants for hairy root induction are tomato and cowpea. Among the *A. rhizogenes* strains, LBA - 9402 induced highest rooting followed by A_4 and ATCC - 15834.

Root induction by *A. rhizogenes* (Strain LBA-9402) with different plant organs (explants) of host plants :

Different parts of tomato, cowpea, carrot and green gram were used for the hairy root induction like leaf, leaf petiole without leaf blade and stem portions. The

Table 7 : Screening of different host explants for callus induction by *Agrobacterium rhizogenes* (Strain ATCC - 15834) on different tissue culture media

Host explants	Callus induction (%)					
	Tissue culture media					
	MS	WM	MWM	B_5	NM	MEAN
A) *Mentha piperita* (Mint)	4.00 (0.81)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	1.60
B) *Mentha citrata* (Mint)	12.00 (1.01)	0.00 (0.71)	8.00 (0.91)	0.00 (0.71)	0.00 (0.71)	4.00
C) *Ocimum gratissimum* (Basil)	4.00 (0.81)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	1.60
D) *Adhatoda vasica* (Vasaca)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.80
E) *Coleus aromaticus* (Coleus)	20.00 (1.16)	4.00 (0.81)	8.00 (0.91)	16.00 (1.11)	12.00 (1.01)	12.00
F) *Vigna unguiculata* (Cowpea)	36.00 (1.50)	4.00 (0.81)	12.00 (1.01)	12.00 (1.01)	20.00 (1.16)	16.80
G) *Vigna radiata* (Green gram)	12.00 (1.01)	8.00 (0.91)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	4.80
H) *Capsicum annum* (Capsicum)	12.00 (1.01)	4.00 (0.81)	8.00 (0.91)	0.00 (0.71)	0.00 (0.71)	4.80
I) *Daucus carota* (Carrot)	24.00 (1.26)	4.00 (0.81)	20.00 (1.16)	4.00 (0.81)	0.00 (0.71)	10.40
J) *Lycopersicon esculentum* (Tomato)	48.00 (1.69)	8.00 (0.91)	24.00 (1.26)	4.00 (0.81)	8.00 (0.91)	18.40
Mean	17.60	3.20	9.20	3.60	4.00	

	SEM ±	CD (P=0.05)
Host plants	0.05	0.13
Media	0.03	0.09
Interaction	0.10	0.29
CV %	26.91	

* Values in parenthesis are transformed values.

MS - Murashige and Skoog medium
WM - White's medium
MWM - Modified White's medium
B_5 - Gamborg B_5 medium
NM - Nitch medium

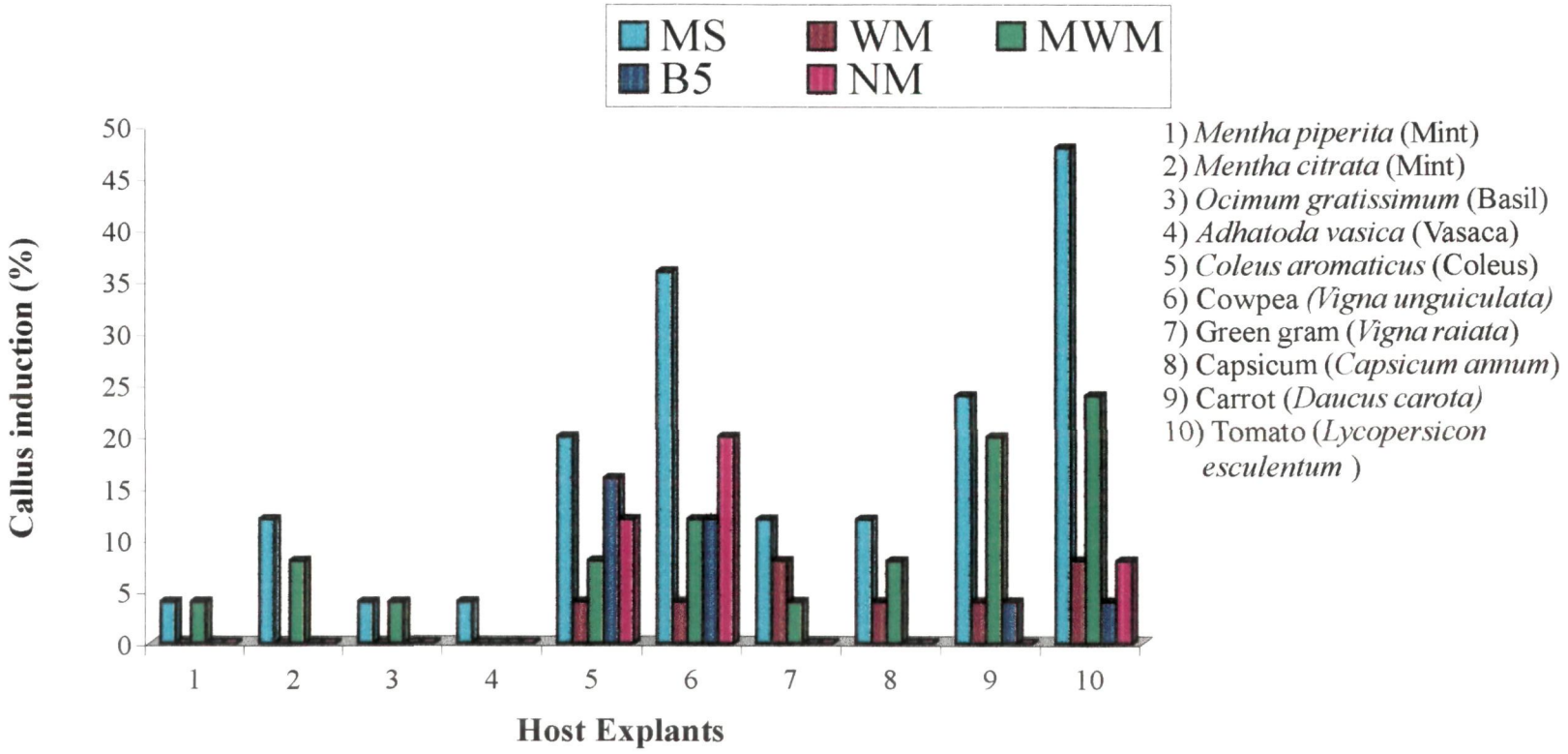


Fig. 3 : Screening of different host explants for callus induction by Agrobacterium rhizogenes (Strain ATCC - 15834) on different tissue culture media

Table 8 : Screening of different host explants for callus induction by *Agrobacterium rhizogenes* (Strain - A_4) on different tissue culture media

Host explants	Callus induction (%)					
	Tissue culture media					
	MS	WM	MWM	B_5	NM	MEAN
A) *Mentha piperita* (Mint)	16.00 (1.11)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	4.00
B) *Mentha citrata* (Mint)	8.00 (0.91)	0.00 (0.71)	8.00 (0.91)	0.00 (0.71)	0.00 (0.71)	3.20
C) *Ocimum gratissimum* (Basil)	4.00 (0.81)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	1.60
D) *Adhatoda vasica* (Vasaca)	0.00 (0.71)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	0.80
E) *Coleus aromaticus* (Coleus)	8.00 (0.91)	0.00 (0.71)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	2.40
F) *Vigna unguiculata* (Cowpea)	24.00 (1.26)	4.00 (0.81)	16.00 (1.11)	20.00 (1.16)	20.00 (1.16)	16.80
G) *Vigna radiata* (Green gram)	20.00 (1.16)	4.00 (0.81)	12.00 (1.01)	0.00 (0.71)	0.00 (0.71)	7.20
H) *Capsicum annum* (Capsicum)	12.00 (1.01)	4.00 (0.81)	4.00 (0.81)	0.00 (0.71)	0.00 (0.71)	4.00
I) *Daucus carota* (Carrot)	32.00 (1.43)	8.00 (0.91)	4.00 (0.81)	8.00 (0.91)	0.00 (0.71)	10.40
J) *Lycopersicon esculentum* (Tomato)	48.00 (1.69)	4.00 (0.81)	20.00 (1.16)	4.00 (0.81)	4.00 (0.81)	16.00
Mean	17.20	2.80	8.00	3.20	2.40	

	SEM ±	CD (P=0.05)
Host plants	0.04	0.12
Media	0.03	0.08
Interaction	0.09	0.26
CV %	24.89	

* Values in parenthesis are transformed values.

MS - Murashige and Skoog medium
WM - White's medium
MWM - Modified White's medium
B_5 - Gamborg B_5 medium
NM - Nitch medium

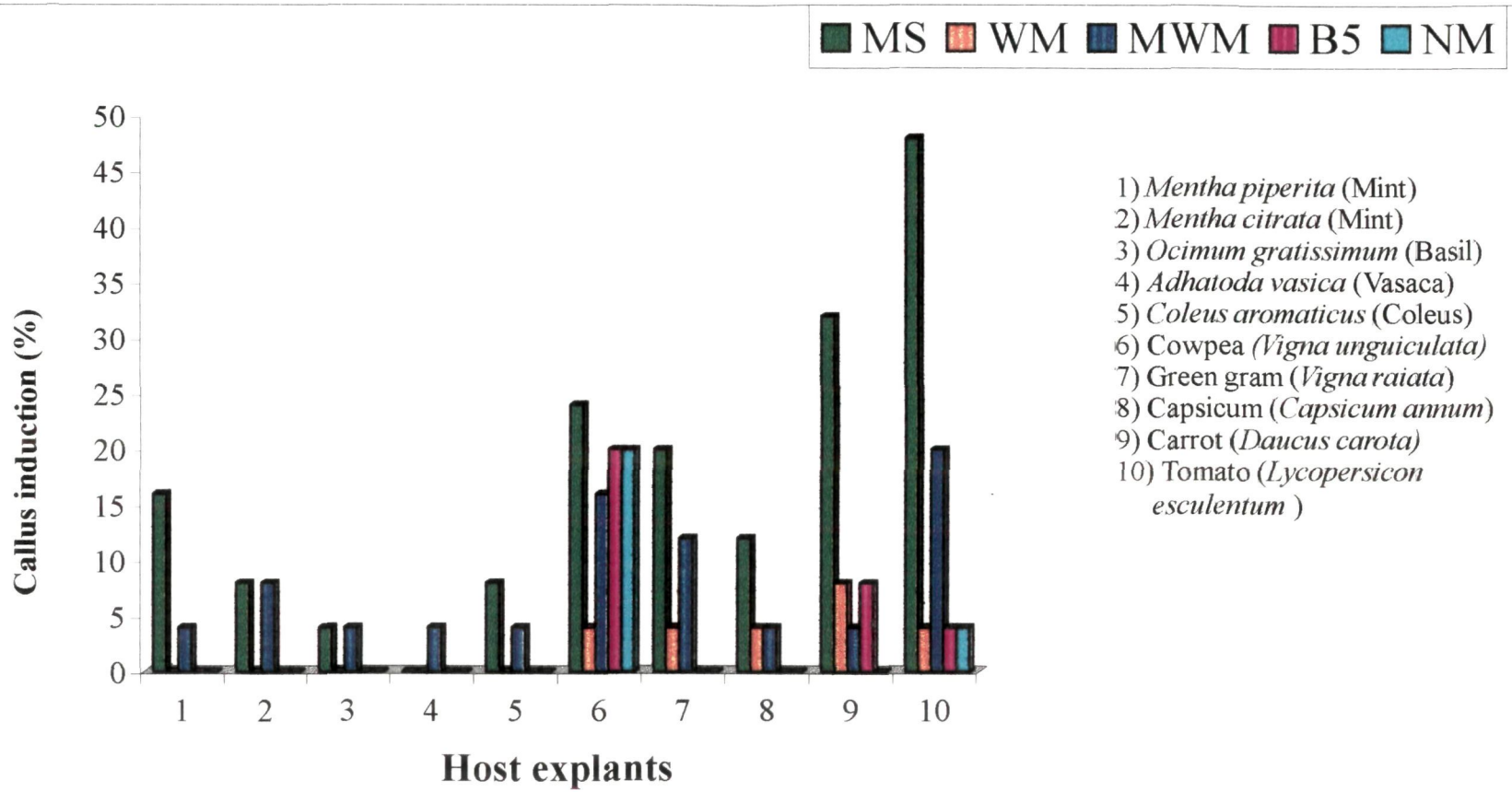


Fig. 4: Selection of different host explants for callus induction by *Agrobacterium rhizogenes* (Strain - A_4) on different tissue culture media

root induction percentage in these explants are given in Table 9 and Fig.5. The results showed that, the per cent rooting in tomato stem explants was highest on 4th day (21%) and reached 24.5 per cent on 16th day. Leaf and petioles responded less (8.7-12.7%), even on 16th day of inoculation. In contrast to this in cowpea, leaf petioles without leaf blade showed highest rooting (20.3%) as compared to stem explants (16.7%). Typical hairy root formation from cowpea leaf petioles is shown in Plate 5. Leaf explants showed the least root induction by *A. rhizogenes* in cowpea and green gram (8.7%). Green gram stem explants showed least root induction (13.0%) compared to cowpea (16.7%) and tomato (24.5%). The statistical analysis of plant materials also shows a significant relation among the plant organs used.

Effect of different levels of sucrose in basal MS medium on the growth of hairy roots :

The experiment was conducted to assess the optimum concentration of sucrose in basal MS medium for hairy root induction. The growth of hairy root recorded on 2nd week with different levels of sucrose is presented in Table 10 and Fig.6. The result brought out that higher root growth rate was induced at 2.5 per cent sucrose level in tomato (6.80 cm), cowpea (4.98 cm) and carrot (3.23 cm). Increase in sucrose level from 2.5 to 4.5 per cent resulted in reduction in root growth. In green gram transformed roots, higher growth was induced in 3.5 per cent sucrose level (3.47 cm) which reduced drastically when the sucrose level was raised to 4.5 per cent (1.90 cm). Statistical analysis also showed

Table 9 : Root induction by *Agrobacterium rhizogenes* (Strain LBA -9402) with different explants of hosts

Explants of hosts	Rooting (%)			
	Days after inoculation			
	4	8	12	16
Tomato (H_1) – Leaf	11.40	12.70	12.70	12.70
Petiole	8.20	8.70	8.70	8.70
Stem	21.20	23.50	23.50	24.50
Cowpea (H_2) – Leaf	8.20	8.70	8.70	8.70
Petiole	17.30	17.30	19.30	20.30
Stem	15.20	15.20	16.70	16.70
Green gram (H_3) – Leaf	8.30	8.30	8.70	8.70
Petiole	7.80	7.80	8.30	8.30
Stem	12.00	12.00	13.00	13.00

	Days	Host plant material	Interaction
H_1 SEM ±	0.063	0.054	0.109
CD (P=0.05)	NS	0.150	NS
H_2 SEM ±	0.071	0.061	0.122
CD (P=0.05)	NS	0.169	NS
H_3 SEM ±	0.039	0.034	0.069
CD (P=0.05)	NS	0.094	NS

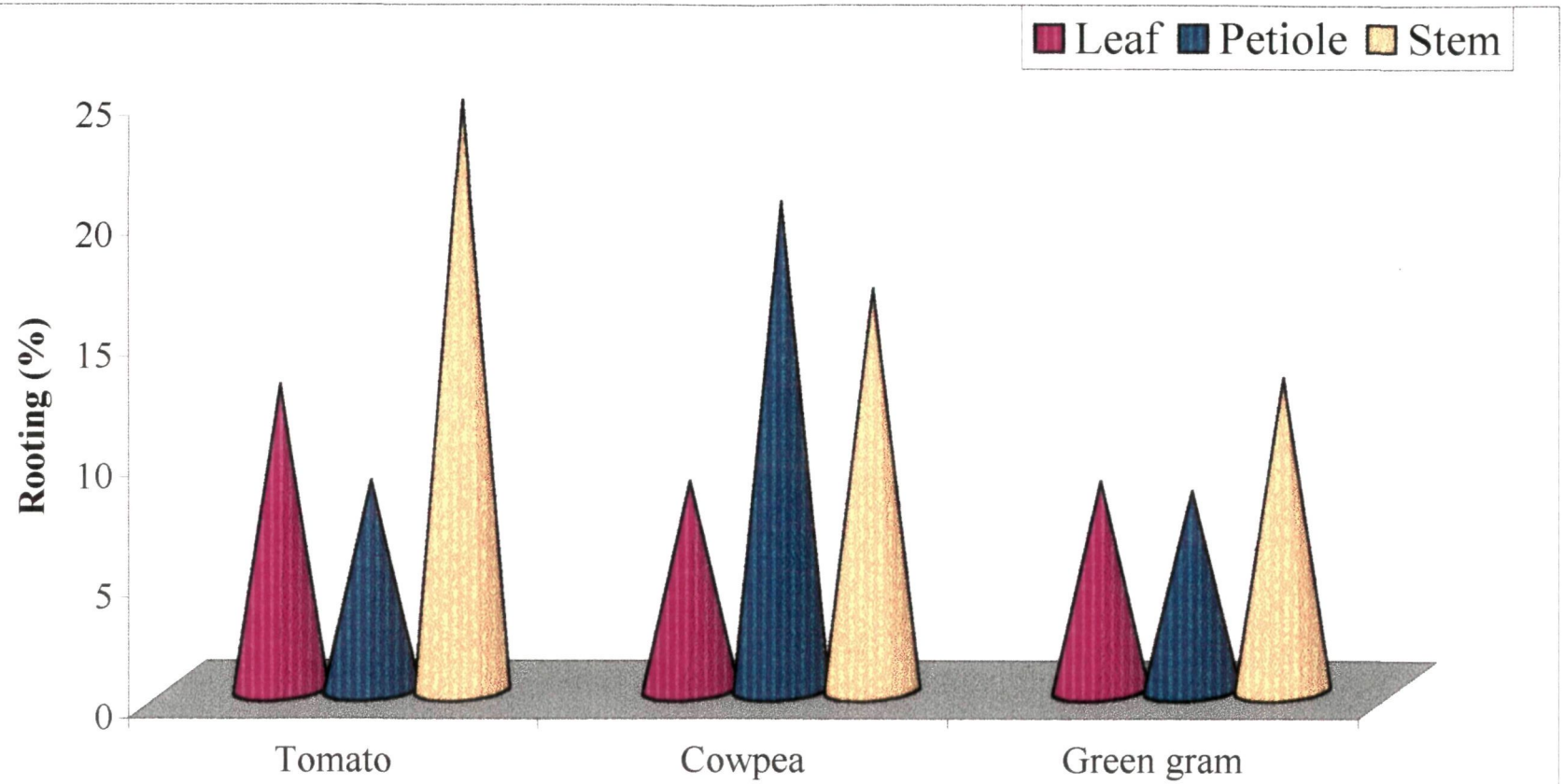


Fig. 5 : Root induction by *Agrobacterium rhizogenes* (Strain LBA - 9402) with different explants of hosts

Table 10 : Effect of different levels of sucrose in MS basal medium on growth of hairy roots

Host	Growth of hairy root (cm) after 2 weeks			
	Sucrose concentration (%)			
	1.50	2.50	3.50	4.50
1. Carrot	2.29	3.23	2.39	1.68
2. Cowpea	2.90	4.98	4.17	3.33
3. Tomato	3.75	6.80	6.28	3.77
4. Green gram	2.04	3.15	3.47	1.90

	Plant material	Sucrose concentration	Interaction
SEM ±	0.12	0.12	0.25
CD (P=0.05)	0.24	0.24	0.49

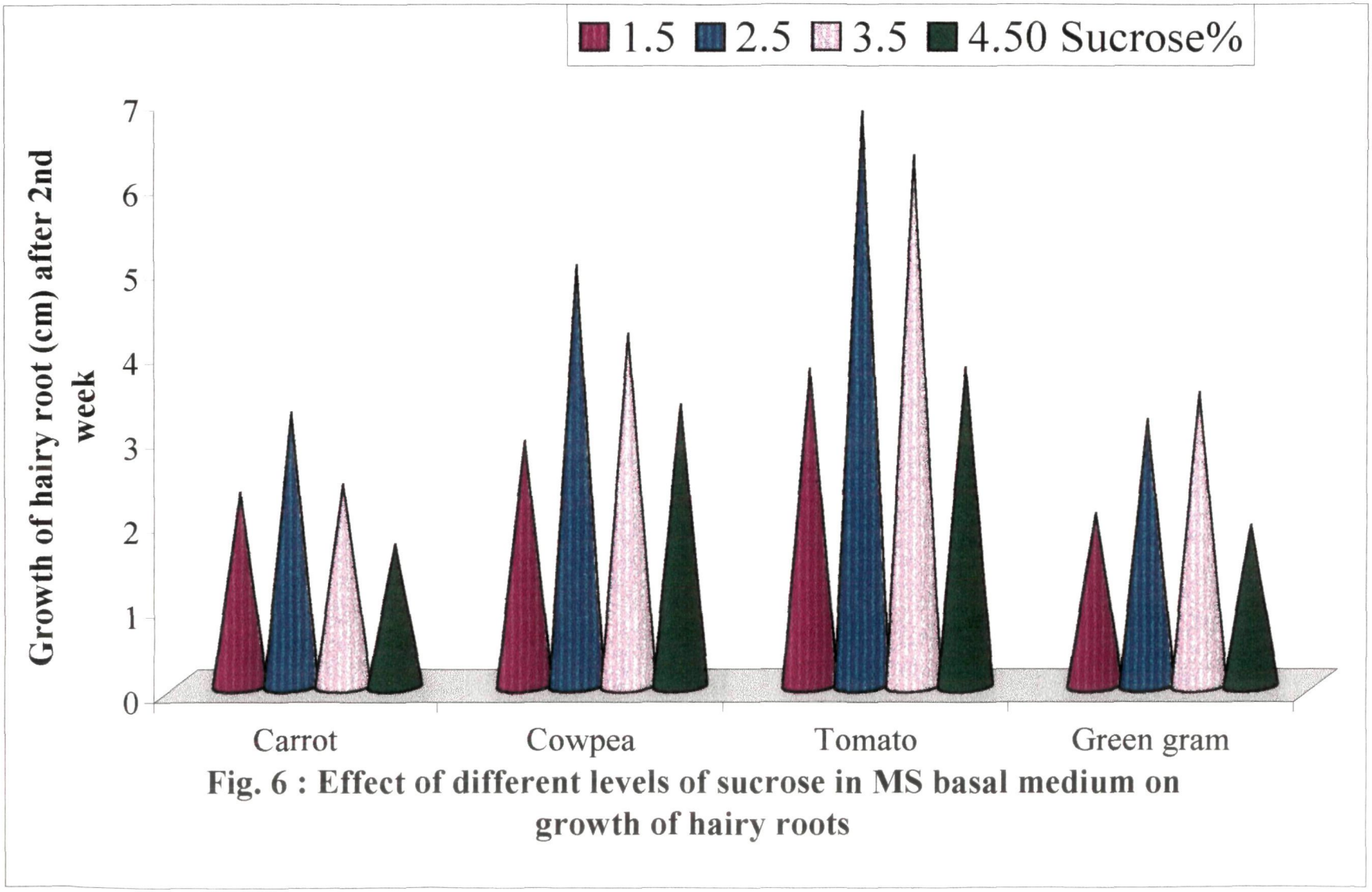


Fig. 6 : Effect of different levels of sucrose in MS basal medium on growth of hairy roots

A

B

PLATE 5: Hairy root formation by *Agrobacterium rhizogenes* (LBA-9402) in cowpea leaf petioles on 4th (A) and 6th day (B)

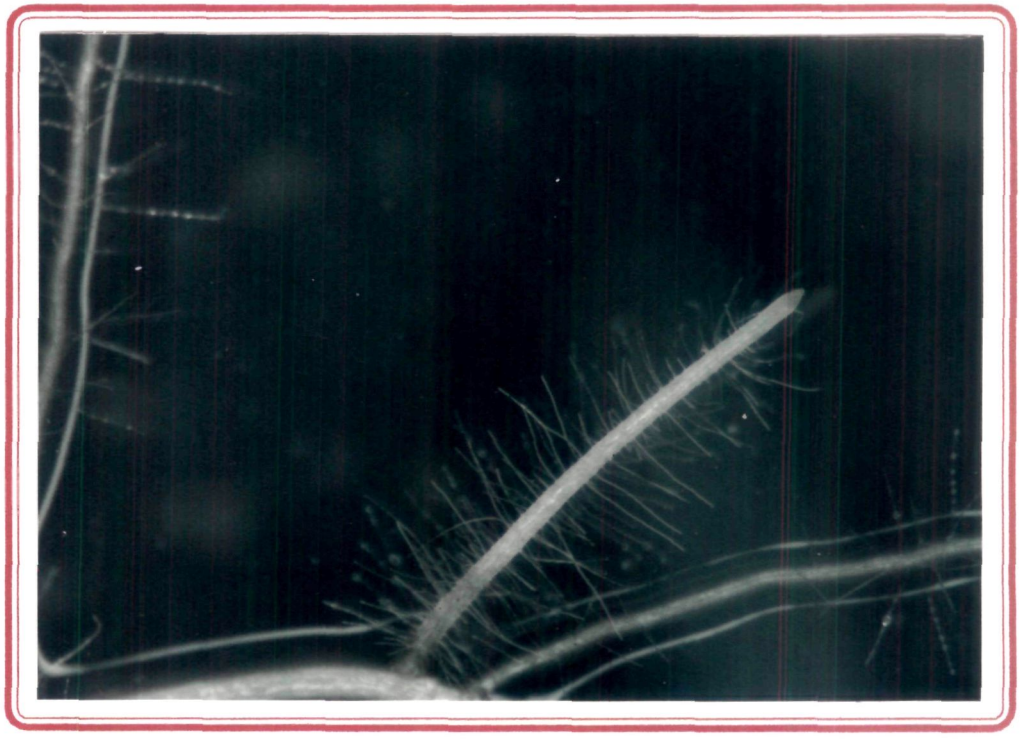
significant interaction between the concentration of sucrose used and the host material used.

Establishment of transformed hairy root :

The co-cultured explants of tomato responded very well to *A. rhizogenes*. Among the ten wild strains of *A. rhizogenes* tested, strains LBA – 9402, ATCC – 15834 and A4 were selected for the continuous culture of transformed roots with tomato. Initial growth of typical hairy roots were observed and the morphological appearance was compared with non-transformed control roots (Plate 6). It was observed that transformed roots had lot of root hairs, which were thicker and grew very fast as compared to non-transformed control roots which had linear growth with less root hairs (Plate 6). After 3rd week of root initiation, transformed roots of tomato had covered almost half of the petridish. Cowpea roots were thicker than tomato roots and it required more time to grow compared to tomato (Plate 7).

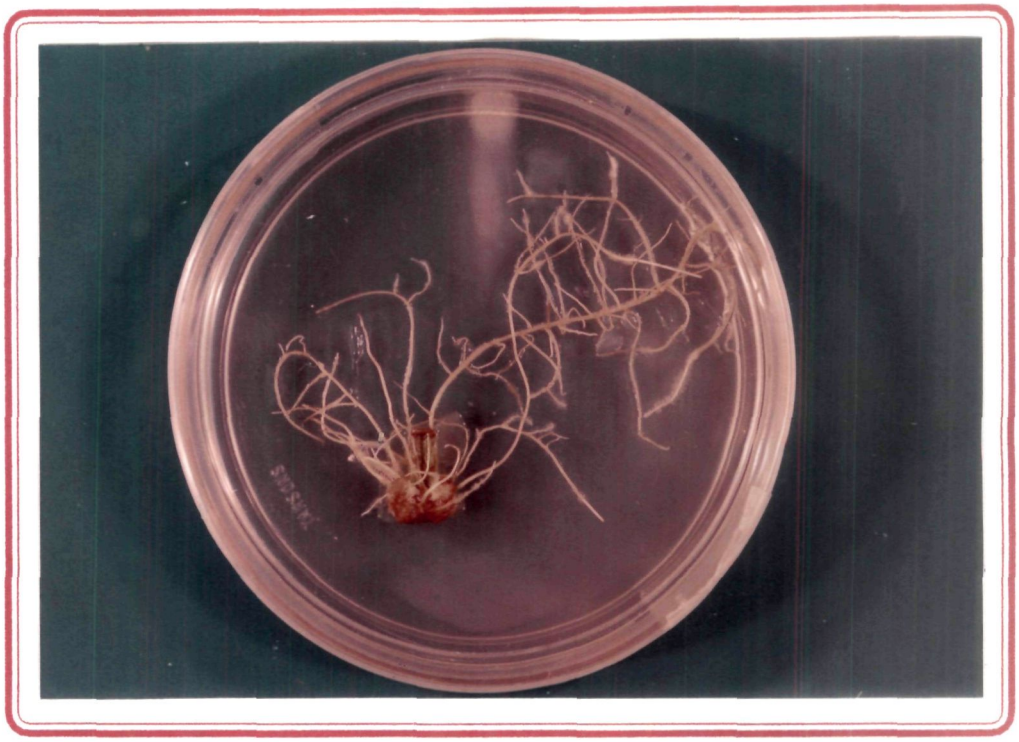
Establishment of transformed roots from tomato was much easier and faster compared to all other host plants studied. It was observed that hairy root induced from tomato stem covered almost the whole petriplate in a month's time (Plate 8). Microscopic observation of these transformed roots showed a clear morphological difference having heavy growth of root hairs which were thicker in size. Non-transformed control roots were thin and produced few root hairs (Plate 9).

A

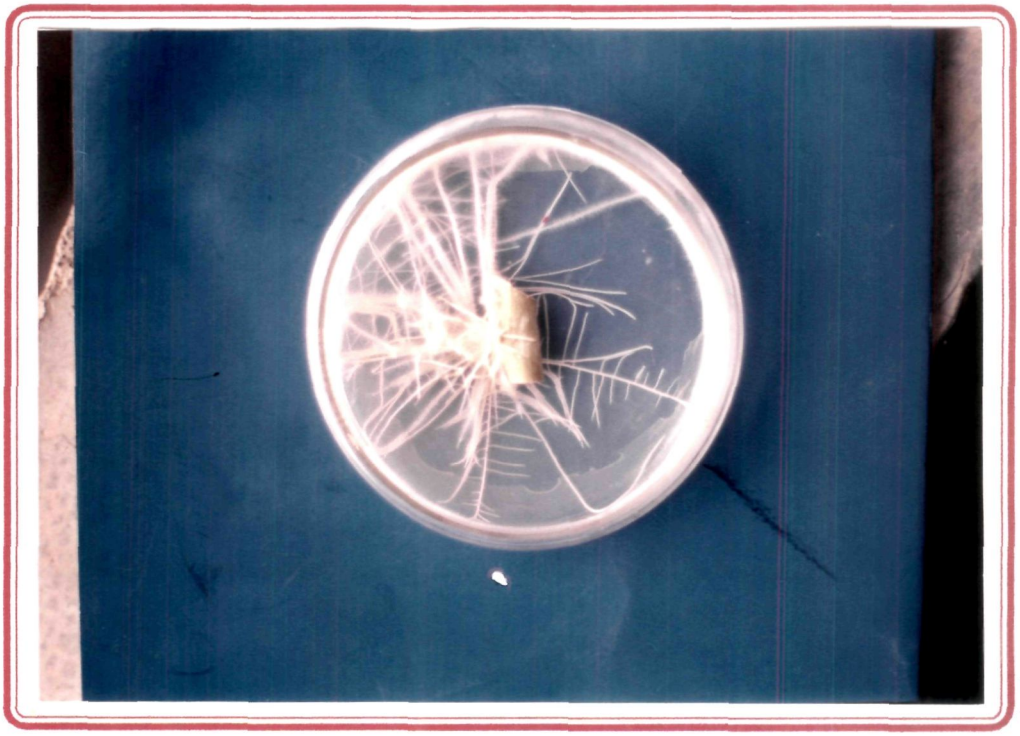


B

PLATE 6: Hairy rooting of tomato stem by *Agrobacterium rhizogenes* strain LBA-9402 (A) and Control explants (B)

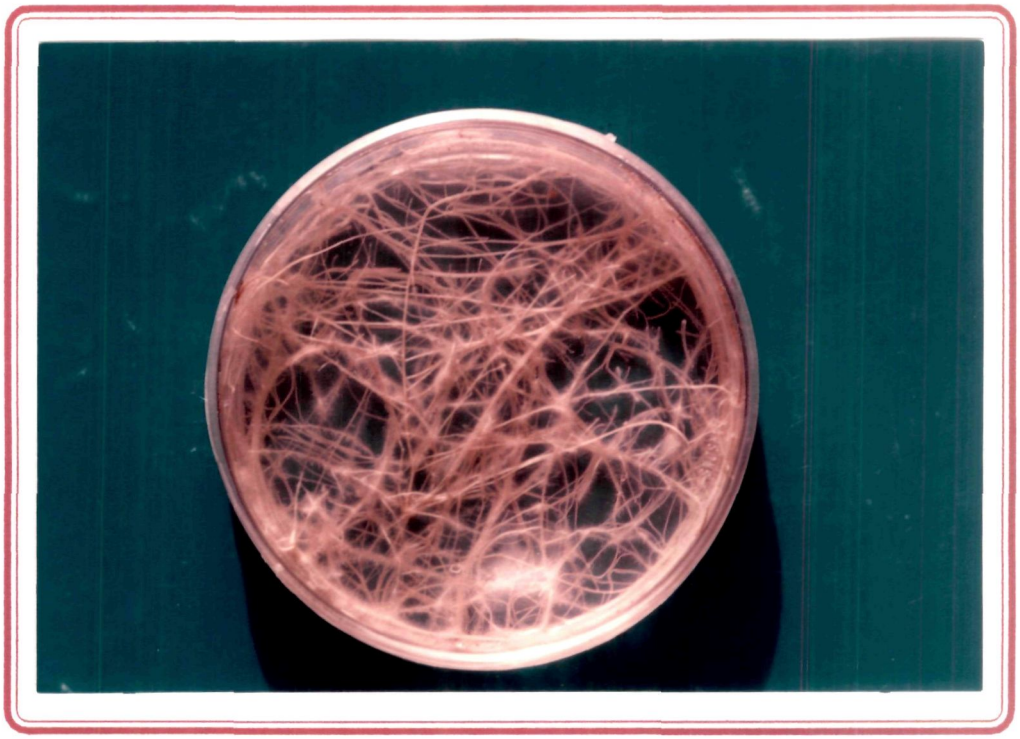


A

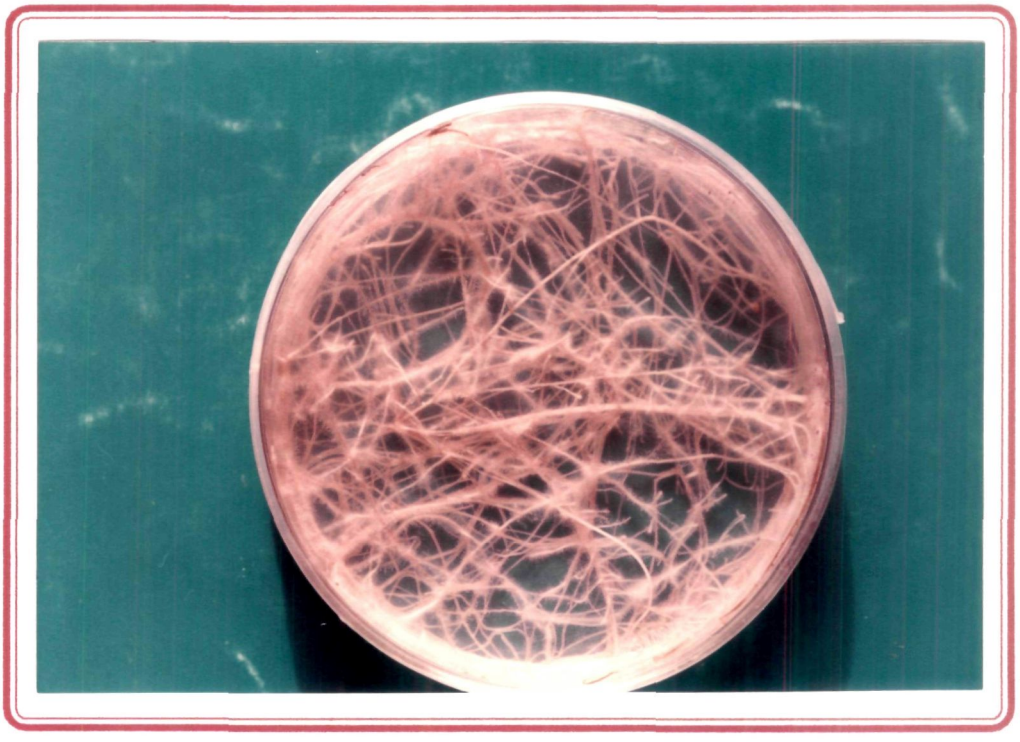


B

PLATE 7: Hairy root after 3 weeks in cowpea (A) and tomato (B) by *Agrobacterium rhizogenes* (LBA-9402)



A

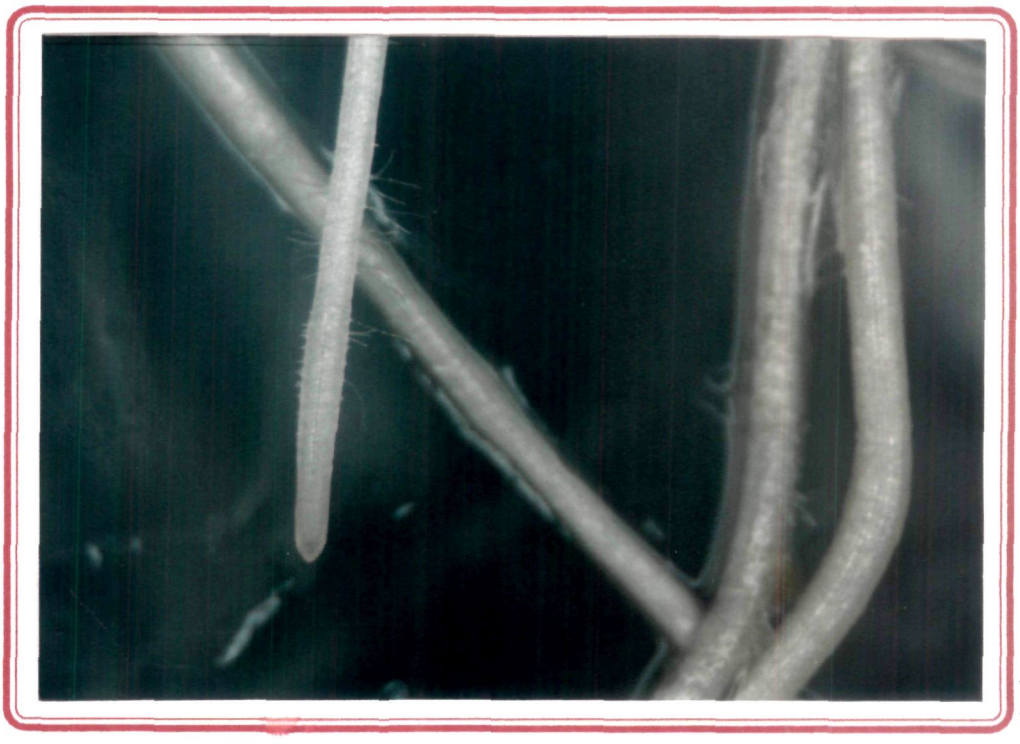


B

PLATE 8: Hairy root establishment after a month by *Agrobacterium rhizogenes* from tomato stem

A. Strain LBA-9402
B. Strain A_4

A



B

PLATE 9: Morphology of hairy roots induced by *Agrobacterium rhizogenes* from tomato stem (A) and non-transformed control (B)

After the formation of transformed roots, these cloned roots were continuously maintained on minimal medium. The roots were further multiplied and grown on fresh media. Pieces of cloned roots (about 1 – 2 cm) were aseptically excised on minimal medium and placed aseptically and the plates were sealed with para-film.

Confirmation of Ri T-DNA transformation by opine assay :

A. rhizogenes after infecting the plant, transfers the Ri – plasmid and produces transformed hairy roots. Thus process of unlimited rooting is by the production of growth hormones by the transformed roots which also produce specific sugar amino acid condensation product called opines. The two major opines produced by *A. rhizogenes* are agropine and mannopine.

To confirm the transfer of Ri T-DNA in hairy roots, root extract from such roots were tested for the production of opines by high voltage electrophoresis method described by Petit *et al.*, 1983. The electrophoretograms thus obtained are presented in Plate 10. The root extracts of three strains LBA – 9402, ATCC – 15834 and A_4 were spotted with two standards agropine and mannopine from Institut Des Sciences Vegetales, Cedex, France. The results confirm the transformation of Ri-plasmid cloned roots showing equivalent positive spots of all the three strains to agropine standard. The silver nitrate stain feebly matched with mannopine. The non-transformed control root extract did not show any spot

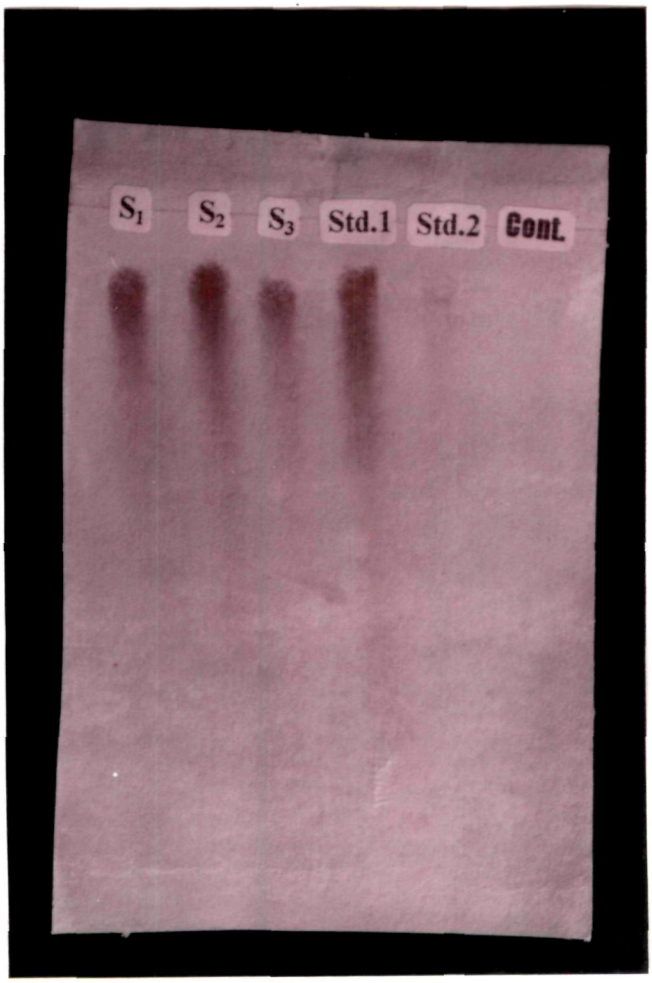
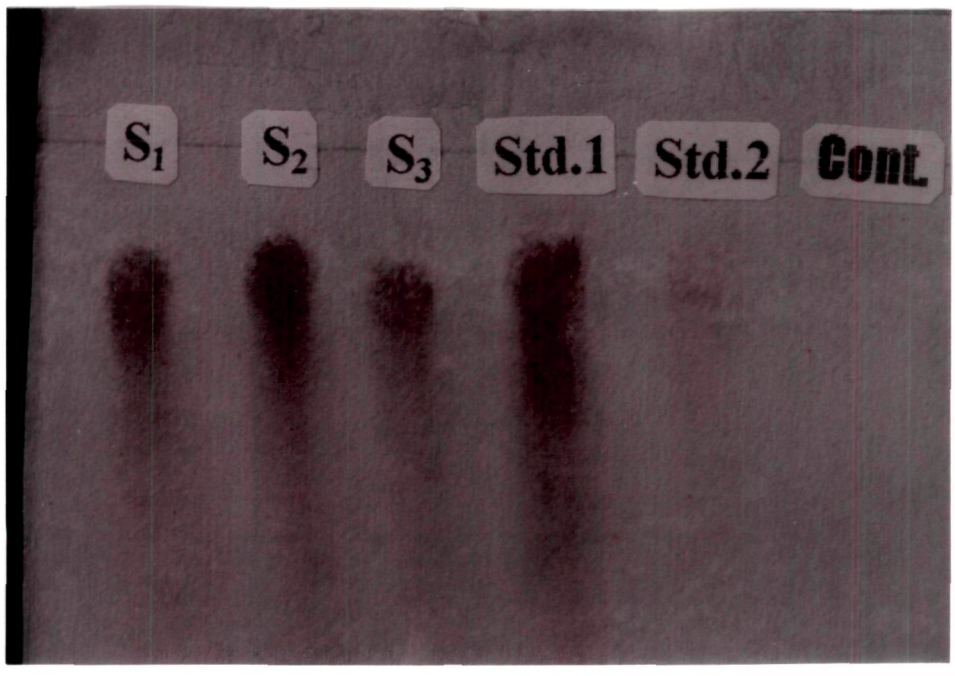


PLATE: 10 Opine assay.
Transformed root extracts :
S_1 - strain LBA-9402
S_2 - strain ATCC-15834
S_3 - strain A_4
Std.- 1 : Standard Agropine
Std.- 2 : Standard Mannopine
Cont. : Non-transformed root sample



matching with either agropine or mannopine confirming non-transformation of control roots.

Establishment and growth of cloned roots of *A. rhizogenes* on different tissue culture media :

The growth of transformed hairy root by *A. rhizogenes* strain LBA – 9402 on different tissue culture media at 25^{0}C is presented in Table 11 and Fig.7. The result revealed that the growth after 20 days is faster on minimal medium (mean 16.47 cm) followed by half MS medium (14.32 cm). The MS basal medium used in the initial establishment of hairy roots did not favour faster growth (10.19 cm). The least growth was in modified White's medium (7.99 cm). On minimal medium the growth of transformed roots was nearly 8 times more compared to the non-transformed control roots. Statistical analysis also showed a significant interaction between the media used and the days.

The growth of transformed roots produced by *A. rhizogenes* strain ATCC – 15834 at 25^{0}C on different tissue culture media is presented in Table 12 and Fig.8. Maximum growth was seen on minimal medium (25.13 cm) after 20 days of incubation. Initial growth (3.72 cm) was also high on minimal medium compared to control (0.56 cm) on 5th day of infection. Half MS medium was the next best showing 16.90 cm growth on 20th day. Modified White's medium did not help the growth of roots. The mean values of the growth of transformed

Table 11 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain LBA - 9402) on different tissue culture media at 25^0C

Medium used	Growth of hairy root (cm) on day				
	5	10	15	20	Mean
Murashige and Skoog medium	2.40	5.69	12.47	20.22	10.19
0.5 x Murashige and Skoog medium	3.37	7.73	16.93	29.26	14.32
Modified White's medium	2.20	4.62	9.36	15.80	7.99
Minimal medium	4.11	10.18	19.62	31.98	16.47
Control (non-transformed roots in MS medium)	0.50	2.02	3.61	5.43	2.89

	SEM ±	CD (P=0.05)
Days	0.12	0.34
Media	0.14	0.38
Interaction	0.28	0.77
CV %	6.06	

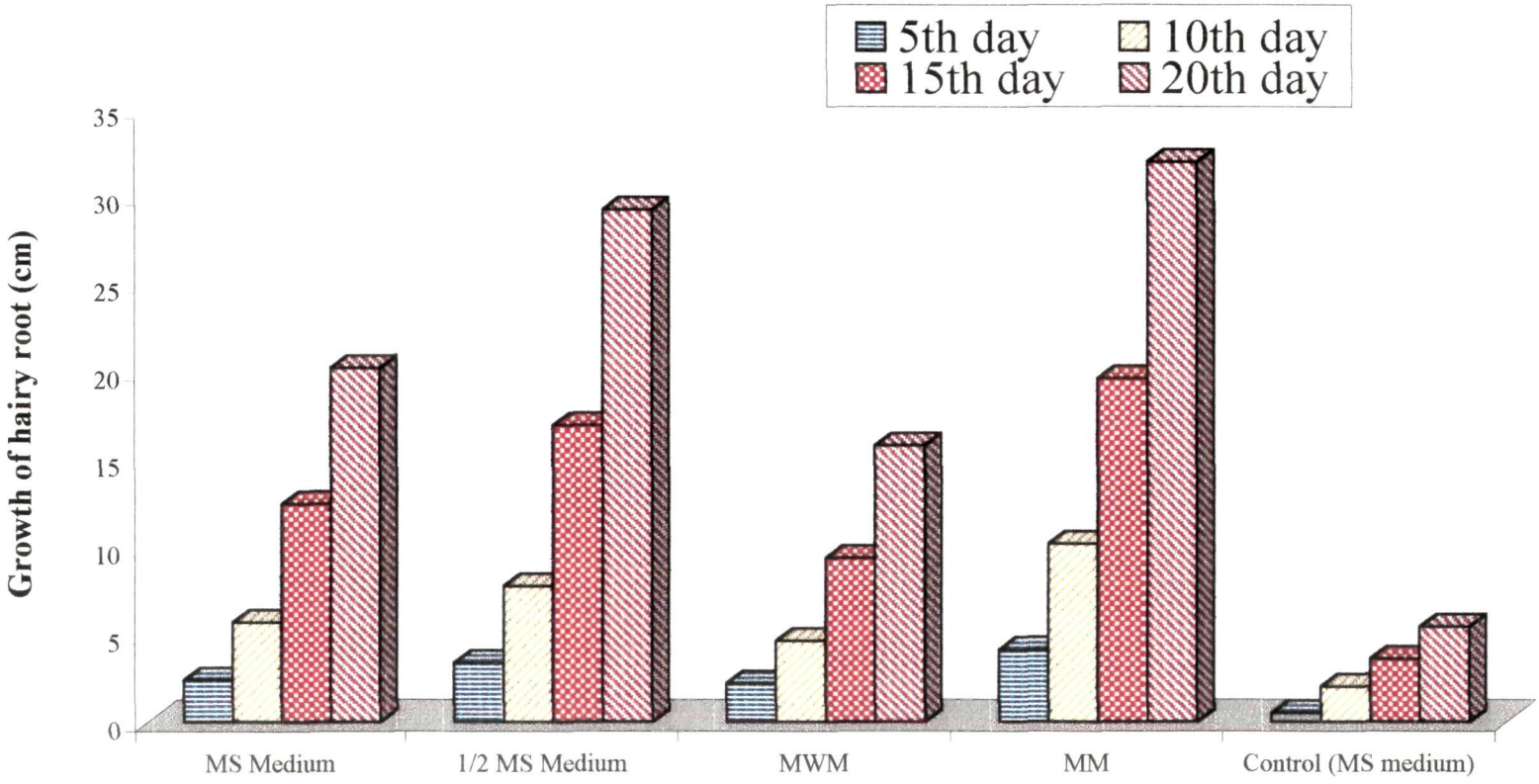


Fig. 7 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain LBA - 9402) on different tissue culture media at 25^0C

Table 12 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain ATCC - 15834) on different tissue culture media at 25^{0}C

Medium used	Growth of hairy root (cm) on day				
	5	10	15	20	Mean
Murashige and Skoog medium	1.85	4.29	9.11	13.91	7.29
0.5 x Murashige and Skoog medium	2.55	5.40	11.70	16.90	9.14
Modified White's medium	1.85	3.59	6.04	8.59	5.02
Minimal medium	3.72	7.33	15.92	25.13	13.03
Control (non-transformed roots in MS medium)	0.56	1.96	3.62	5.64	2.95

	SEM ±	CD (P=0.05)
Days	0.28	0.77
Media	0.31	0.86
Interaction	0.62	1.73
CV %	18.71	

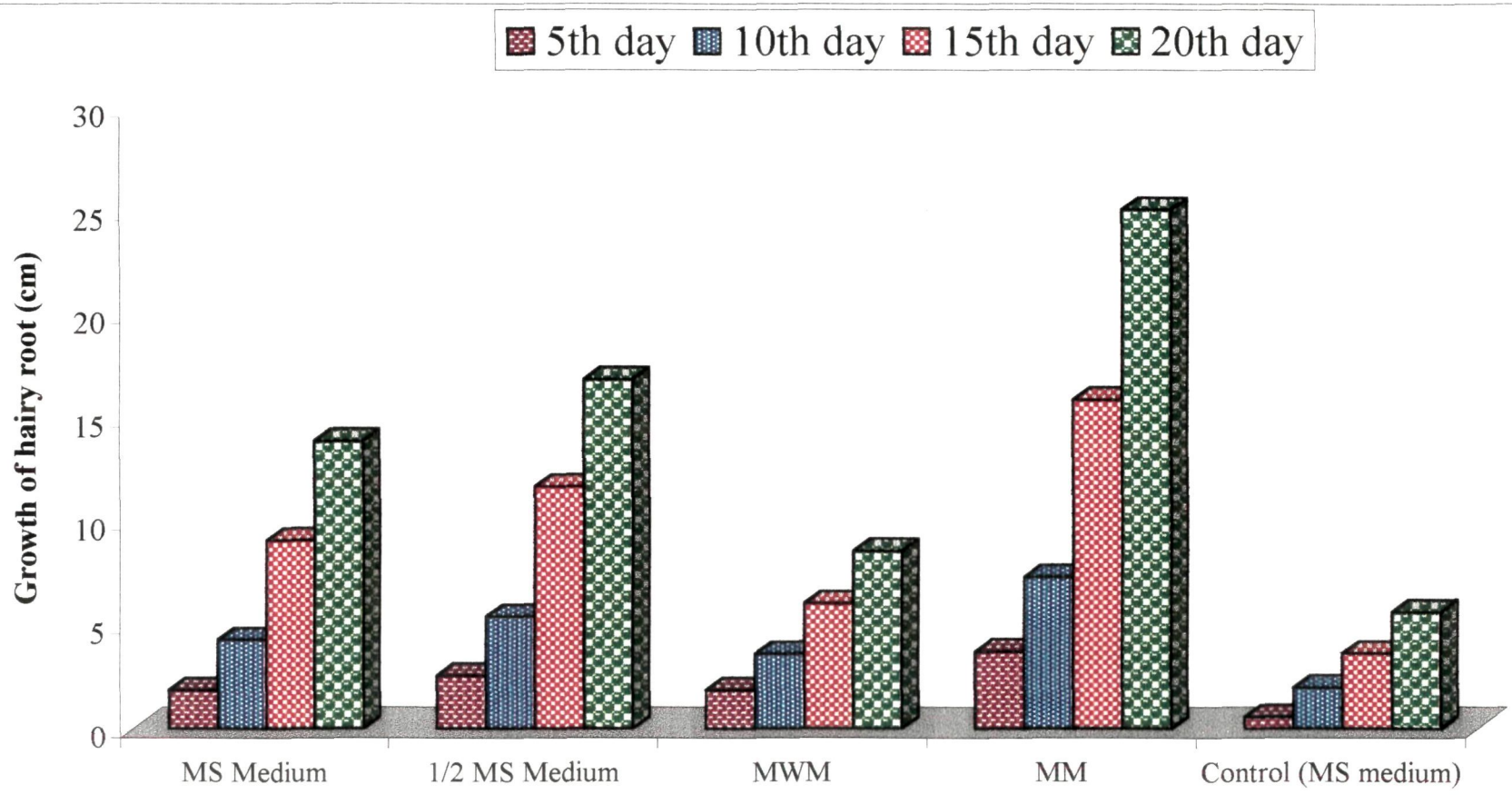


Fig. 8 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain ATCC - 15834) on different tissue culture media at 25^0C

roots on minimal medium was more (13.03 cm) as compared to control roots with only 2.95 cm growth.

A similar experiment conducted using A_4 strain of *A. rhizogenes* is presented in Table 13 and Fig.9. Initial growth of transformed roots up to 5th day was almost similar (2.23 to 3.60 cm) in all the media. But later roots grew faster on minimal medium (30.09 cm) followed by half MS (24.78 cm), MS medium (18.41 cm) and on MWM (13.78 cm), the figures given in parenthesis being the growth recorded 20 days after incubation.

Establishment and growth of transformed roots at 30^{0}C :

All the processes of initial establishment of transformed roots was done at lower temperature of 25^{0}C and at dark. After ten days growth, the transformed roots were incubated at 30^{0}C. The results of such growth at higher temperature are given below.

The growth of hairy root formed by *A. rhizogenes* strain LBA – 9402 incubated at 30^{0}C is given in Table 14 and Fig.10. Both on half MS medium and minimal medium the growth was more. Growth of roots after 20 days on minimal medium was highest (33.70 cm) which almost doubled the growth on the 5th day (4.49 cm). Transformed roots at higher temperature also preferred half MS medium showing almost similar (30 cm) growth as in minimal medium and followed the

Table 13 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain - A_4) on different tissue culture media at 25^0C

Medium used	Growth of hairy root (cm) on day				
	5	10	15	20	Mean
Murashige and Skoog medium	2.23	5.26	11.41	18.41	9.32
0.5 x Murashige and Skoog medium	2.80	6.73	14.18	24.78	12.12
Modified White's medium	2.03	4.58	8.34	13.78	7.18
Minimal medium	3.60	9.18	18.29	30.09	15.29
Control (non-transformed roots in MS medium)	0.50	1.96	3.56	5.47	2.87

	SEM ±	CD (P=0.05)
Days	0.20	0.56
Media	0.22	0.63
Interaction	0.45	1.26
CV %	10.95	

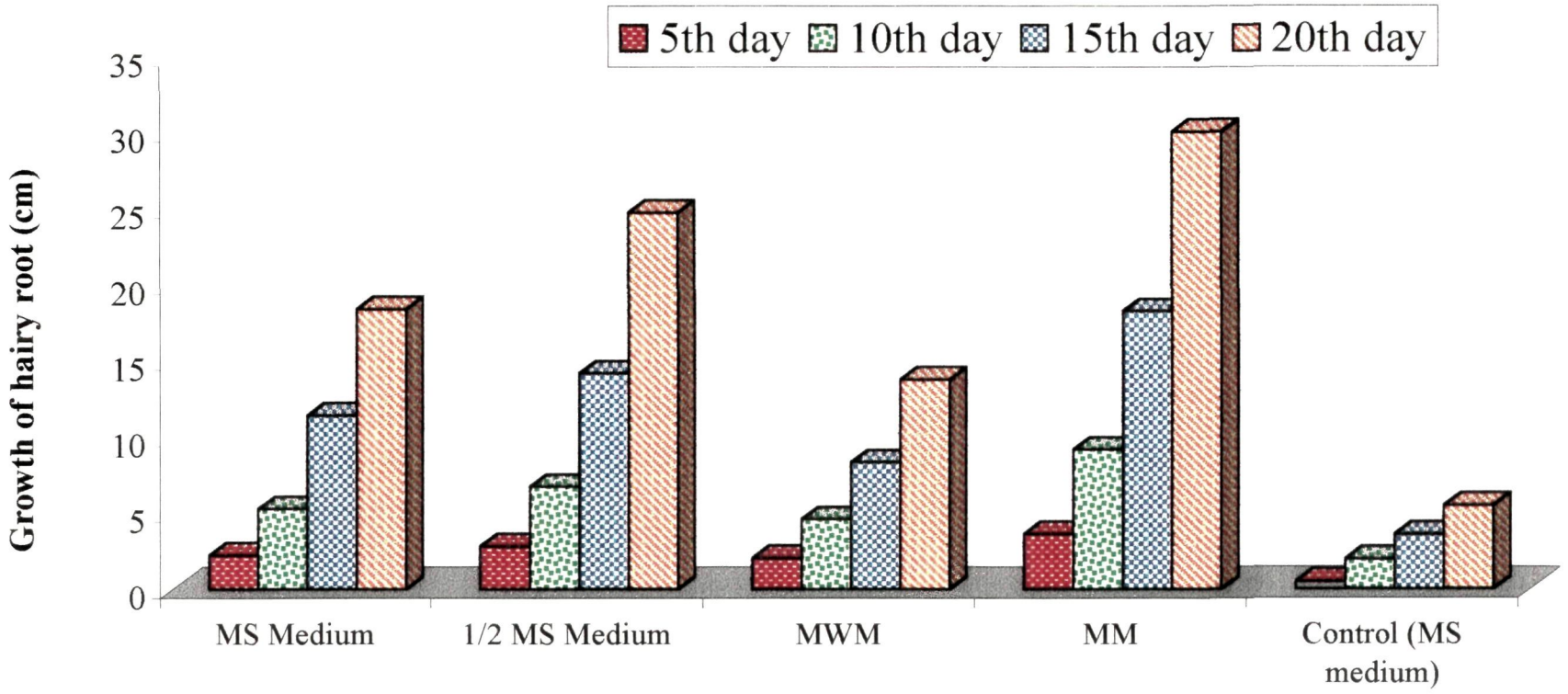


Fig. 9 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain - A4) on different tissue culture media at 25^0C

Table 14 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain LBA - 9402) on different tissue culture media at 30^0C

Medium used	Growth of hairy root (cm) on day				
	5	10	15	20	Mean
Murashige and Skoog medium	2.38	5.99	12.85	19.15	10.09
0.5 x Murashige and Skoog medium	3.95	8.83	18.86	30.11	15.43
Modified White's medium	2.09	4.69	8.86	14.33	7.49
Minimal medium	4.49	11.22	21.15	33.70	17.64
Control (non-transformed roots in MS medium)	0.47	2.03	3.73	5.85	3.02

	SEM ±	CD (P=0.05)
Days	0.17	0.47
Media	0.19	0.53
Interaction	0.38	1.07
CV %	8.05	

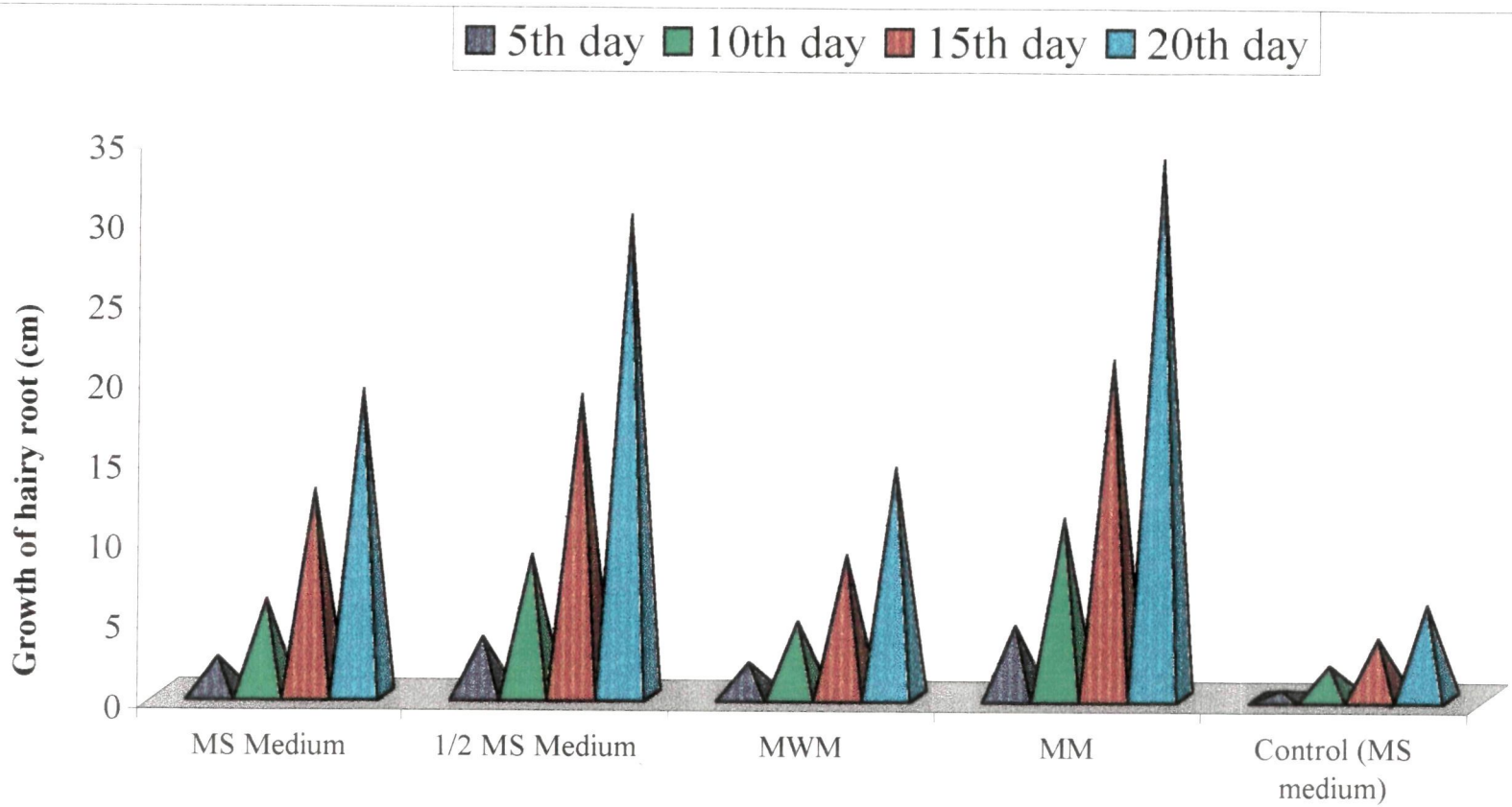


Fig. 10 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain LBA - 9402) on different tissue culture media at 30^0 C

same trend of growth. Modified White's medium did not support good root growth (14.35 cm) after 20 days compared to half MS and minimal media.

Establishment and growth of hairy roots formed from strains ATCC - 15834 and A_4 on different tissue culture media at 30^0 C are given in Tables 15 and 16 and Figs.11 and 12.

Cloned roots from *A. rhizogenes* strain ATCC - 15834 showed highest growth on minimal medium on 20^{th} day (30.11 cm) which was 10 fold more when compared to the 5^{th} day growth of 3.69 cm. On half MS medium the growth was 2.90 cm on 5^{th} day and 29.03 cm on 20^{th} day of incubation. Growth on other media viz., MS medium (20.84 cm) and MWM (16.02 cm) were moderate. Non- transformed roots had the least growth of only 2.82 cm.

The growth of transformed roots induced by A_4 strain of *A rhizogenes* at 30^0C is presented in Table 16 and Fig.12. A similar result of faster growth was seen on minimal medium on 20^{th} day (32.16 cm) followed by growth on half MS medium (30.00 cm). Growth on MS medium was 20.12 cm and that on MWM was 16.27 cm after 20 days incubation. Growth on MWM was the least among transformed roots.

Table 15 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain ATCC - 15834) on different tissue culture media at 30^{0}C

Medium used	Growth of hairy root (cm) on day				
	5	10	15	20	Mean
Murashige and Skoog medium	2.60	6.34	12.94	20.84	10.68
0.5 x Murashige and Skoog medium	2.90	7.78	16.38	29.03	14.52
Modified White's medium	2.20	4.96	9.49	16.02	8.16
Minimal medium	3.69	9.86	19.91	30.11	15.89
Control (non-transformed roots in MS medium)	0.50	1.83	3.46	5.51	2.82

	SEM ±	CD (P=0.05)
Days	0.13	0.36
Media	0.14	0.40
Interaction	0.29	0.81
CV %	6.45	

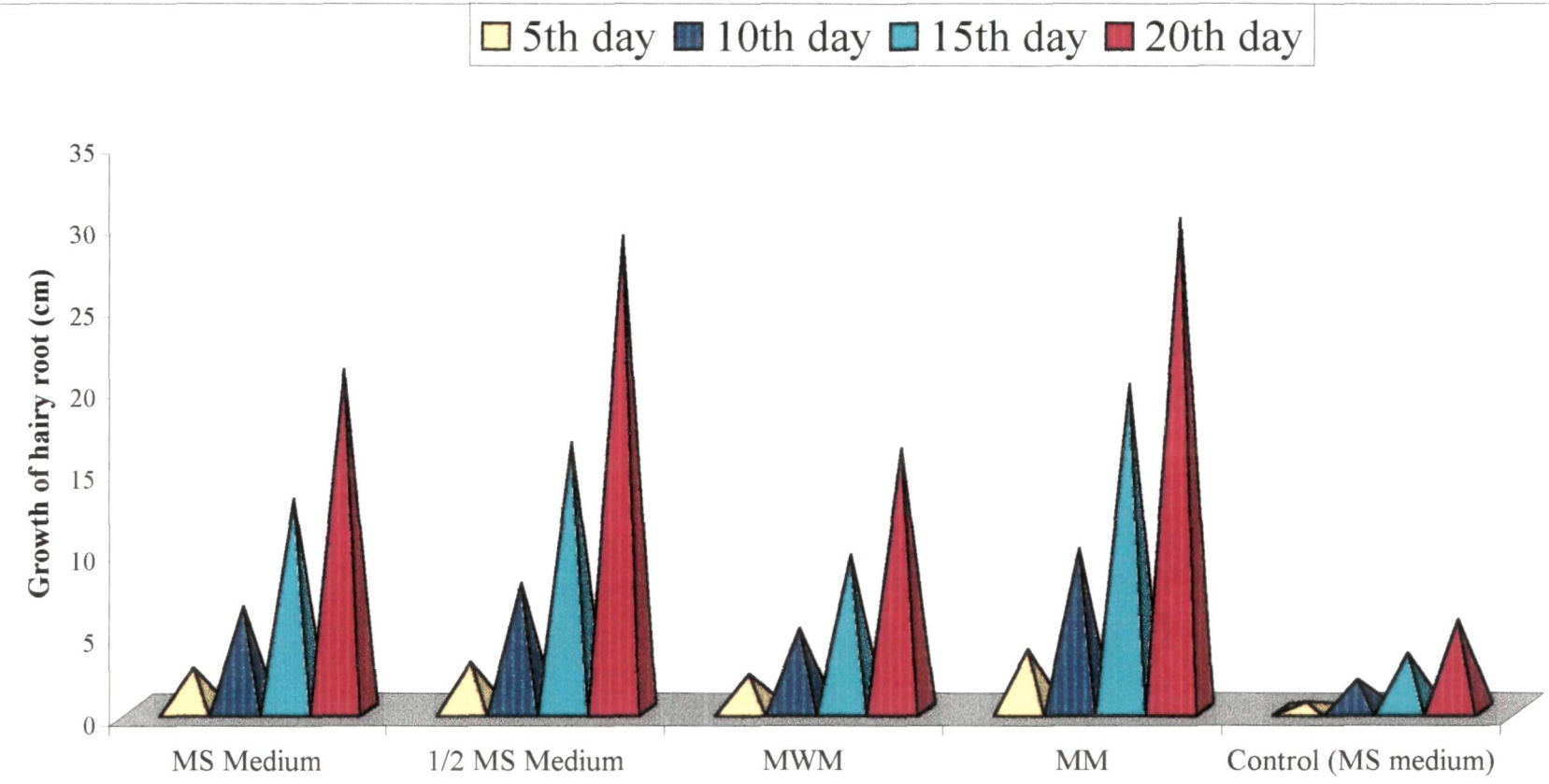


Fig. 11 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain ATCC - 15834) on different tissue culture media at 30^0 C

Table 16 : Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain - A_4) on different tissue culture media at 30^0C

Medium used	Growth of hairy root (cm) on day				
	5	10	15	20	Mean
Murashige and Skoog medium	2.30	5.66	12.37	20.12	10.11
0.5 x Murashige and Skoog medium	2.82	7.70	17.48	30.00	15.49
Modified White's medium	2.18	5.17	10.17	16.27	8.45
Minimal medium	3.69	10.26	20.66	32.16	16.69
Control (non-transformed roots in MS medium)	0.46	1.85	3.48	5.93	2.93

	SEM ±	CD (P=0.05)
Days	0.12	0.35
Media	0.14	0.39
Interaction	0.28	0.79
CV %	6.15	

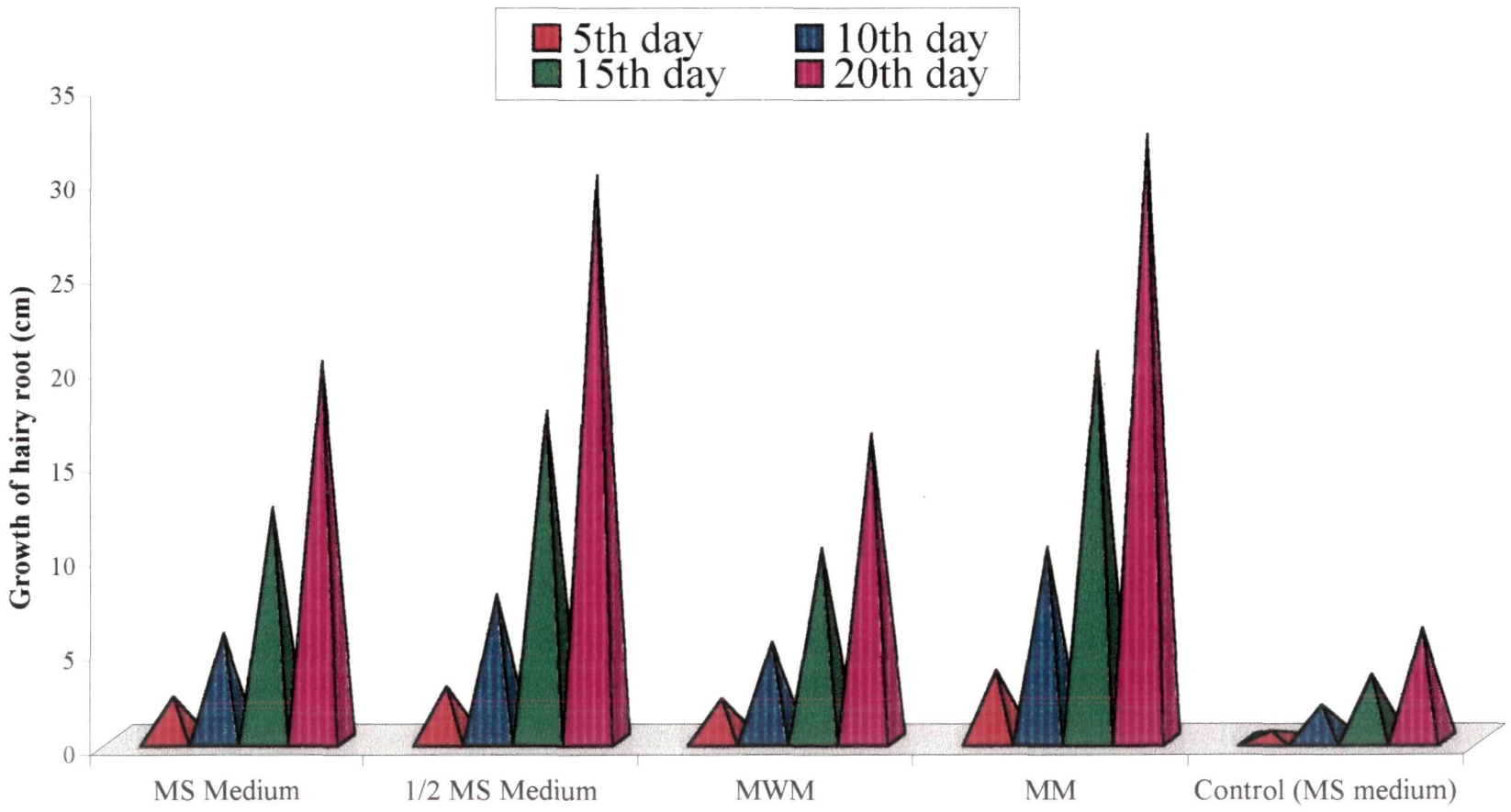


Fig. 12: Establishment of tomato hairy roots by *Agrobacterium rhizogenes* (Strain A_4) on different tissue culture media at 30^0 C

Effect of growth regulators on the growth of transformed tomato roots induced by *A. rhizogenes* (Strain LBA - 9402) :

In most of the tissue culture work growth regulators are added in culture media to maintain the growth of explants. The effect of growth regulators NAA,IBA and IAA on the growth of transformed roots were recorded on 8 and 12 days after incubation at 25^0C. The effect of growth regulators 8 days after incubation is presented in Table 17 and Fig.13.

NAA at concentrations of 0.5, 1.0, 1.5, 2.0 mg/l resulted in root growth of 0.70, 1.10, 0.91 and 0.65 cm respectively. IBA at the lowest concentration of 0.5 mg/l produced better growth (1.06 cm) and while at higher concentrations it retarded root growth. IAA resulted in highest root growth at 1.0 mg/l and least at 0.5 mg/l. Control non-transformed roots had a growth of 1.10 cm 8 days after incubation.

The effect of growth regulators on the growth of transformed roots recorded after 12 days of incubation is given in Table 18 and Fig.14. At 0.5 mg/l concentration, IBA had shown highest root growth of 1.46 cm followed by NAA 0.92 cm and IAA. At 1.0 mg/lconcentration NAA has showed better growth (1.14 cm) compared to other two growth regulators. At 1.5 mg/l concentration, NAA and IBA performed better producing root growth of 1.02 and 1.10 cm respectively while IAA resulted in 0.68 cm root growth. At 2.0 mg/l concentration all the growth

Table 17 : Effect of growth regulators on the growth of tomato hairy roots induced by *Agrobacterium rhizogenes* (Strain LBA – 9402) 8 days after incubation on MS basal medium at 25^0C

Growth regulator	Growth of hairy root (cm)			
	Concentration of growth regulator (mg/l)			
	0.50	1.00	1.50	2.00
Naphthalene - acetic - acid	0.70	1.10	0.91	0.65
Indole - butyric - acid	1.06	0.71	0.66	0.57
Indole - acetic - acid	0.54	0.98	0.68	0.62
Control (without GR)	1.10	1.10	1.10	1.10

	SEM ±	CD (P=0.05)
Growth regulators	0.02	0.07
Concentrations	0.03	0.10
Interaction	0.06	0.17
CV %	19.74	

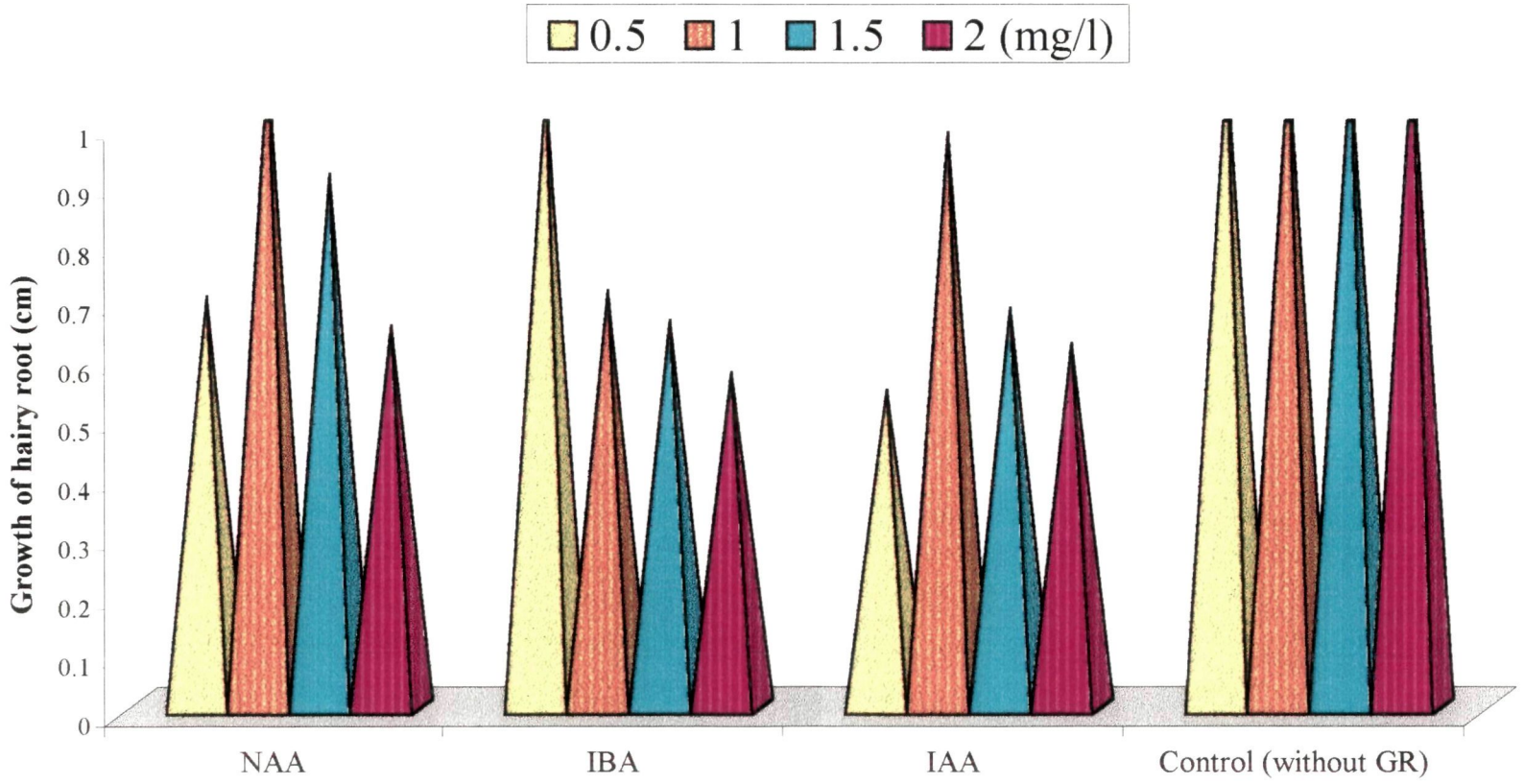


Fig. 13 : Effect of growth regulators on the growth of tomato hairy roots induced by *Agrobacterium rhizogenes* (Strain LBA - 9402) 8 days after incubation on MS basal medium at 25^0 C

Table 18 : Effect of growth regulators on the growth of tomato hairy roots induced by *Agrobacterium rhizogenes* (Strain LBA – 9402) 12 days after incubation on MS basal medium at 25^{0}C

Growth regulator	Growth of hairy root (cm)			
	Concentration of growth regulator (mg/l)			
	0.50	1.00	1.50	2.00
Naphthalene - acetic - acid	0.92	1.14	1.02	0.79
Indole - butyric - acid	1.46	0.95	1.10	0.66
Indole - acetic - acid	0.83	1.35	0.68	0.66
Control (without GR)	2.53	2.53	2.53	2.53

	SEM ±	CD (P=0.05)
Growth regulators	0.03	0.09
Concentrations	0.04	0.12
Interaction	0.07	0.21
CV %	18.54	

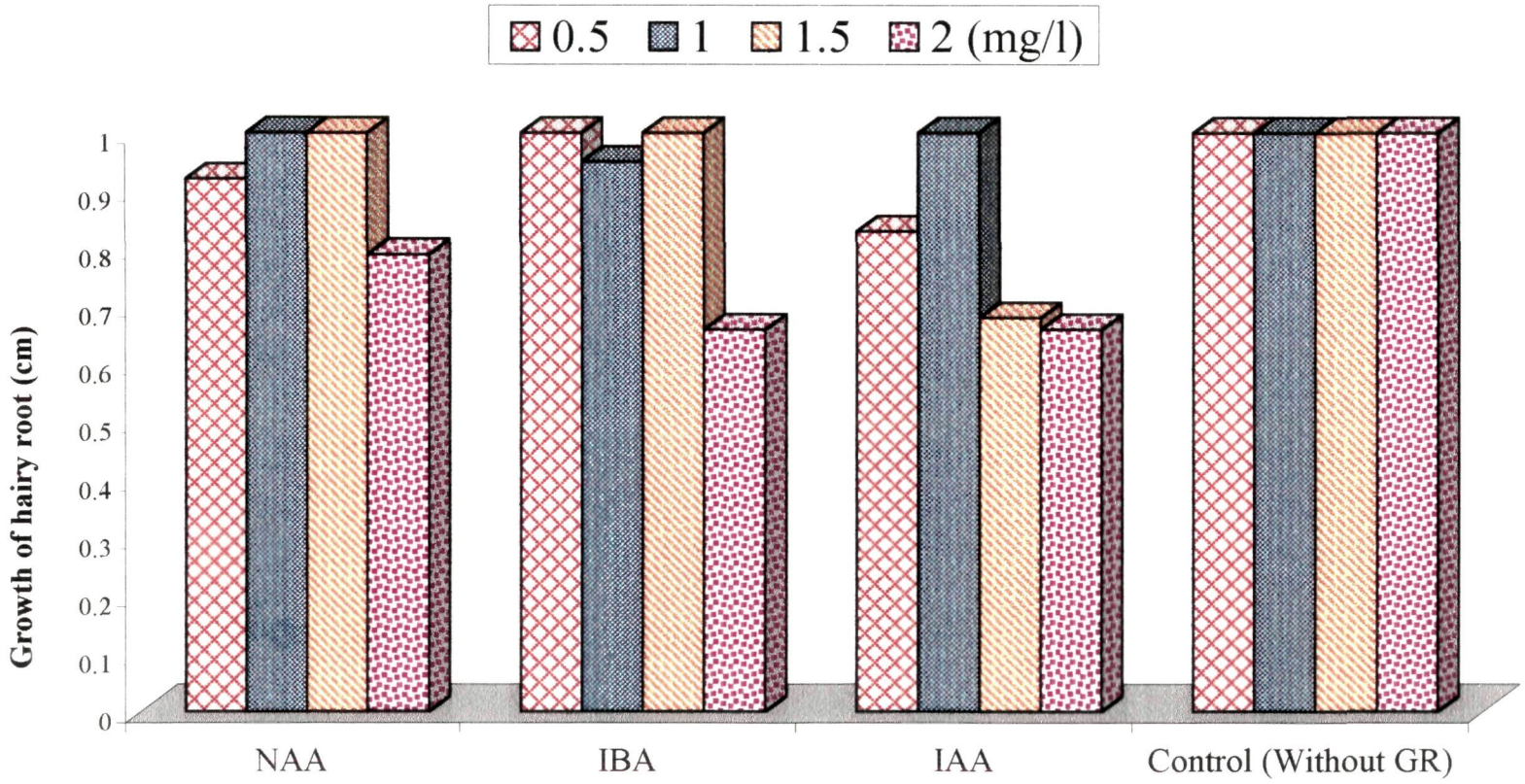


Fig . 14 : Effect of growth regulators on the growth of tomato hairy roots induced by *Agrobacterium rhizogenes* (Strain LBA - 9402) 12 days after incubation on MS basal medium at 25^0 C

regulators had adverse effect and retarded root growth. Untreated control roots grew steadily and showed a growth of 2.53 cm on 12th day of incubation.

It was also observed that, whenever the growth regulator treatments were given most of the roots became stunted with thickened root tips compared to untreated control roots showing healthy and fast growth (Plate 11.).

VAM spore isolation :

The success of dual culturing VAM on transformed roots lies in the isolation and purification of VAM spores used for inoculating transformed roots. The spores of specific VAM culture were isolated by wet sieving and decantation method from 50 g soil explained earlier under material and methods. It is essential to remove debris and other contaminants from the spores. This is usually done by the sucrose gradient method suggested by Furlan *et al.* (1980). It is difficult to store the spores in sucrose solution and hence, removed usually by washing. This many a times leads to contamination of spores. Hence, glycerol as a possible substitute of sucrose was attempted.

VAM spore isolation by modified gradient centrifugation method :

The usual method of using sucrose gradient at 30:60 and 40:80 gradients was compared with the modified gradient method using glycerol at 30:60 and 40:80 gradients. The results are given in Table 19 and Fig.15. The number of *Glomus*

Table 19 : Retrieval of VAM spores by sucrose and glycerol gradient methods

VAM fungus	Spore numbers retrieved/50 g soil				
	Sucrose gradient		Glycerol gradient		Mean
	30 : 60	40 : 80	30 : 60	40 : 80	
Glomus fasciculatum	372.50	363.75	505.25	446.50	422.00
Glomus mosseae	340.00	323.75	373.25	462.50	374.87
Gigaspora margarita	334.25	407.50	393.75	432.50	392.75
Glomus geosporum	261.25	208.00	312.50	266.25	262.00
Mean	315.75	325.75	396.18	401.93	

	SEM ±	CD (P=0.05)
VAM fungi	15.58	44.36
Methods	15.58	44.36
Interaction	31.17	88.72
CV %	17.32	

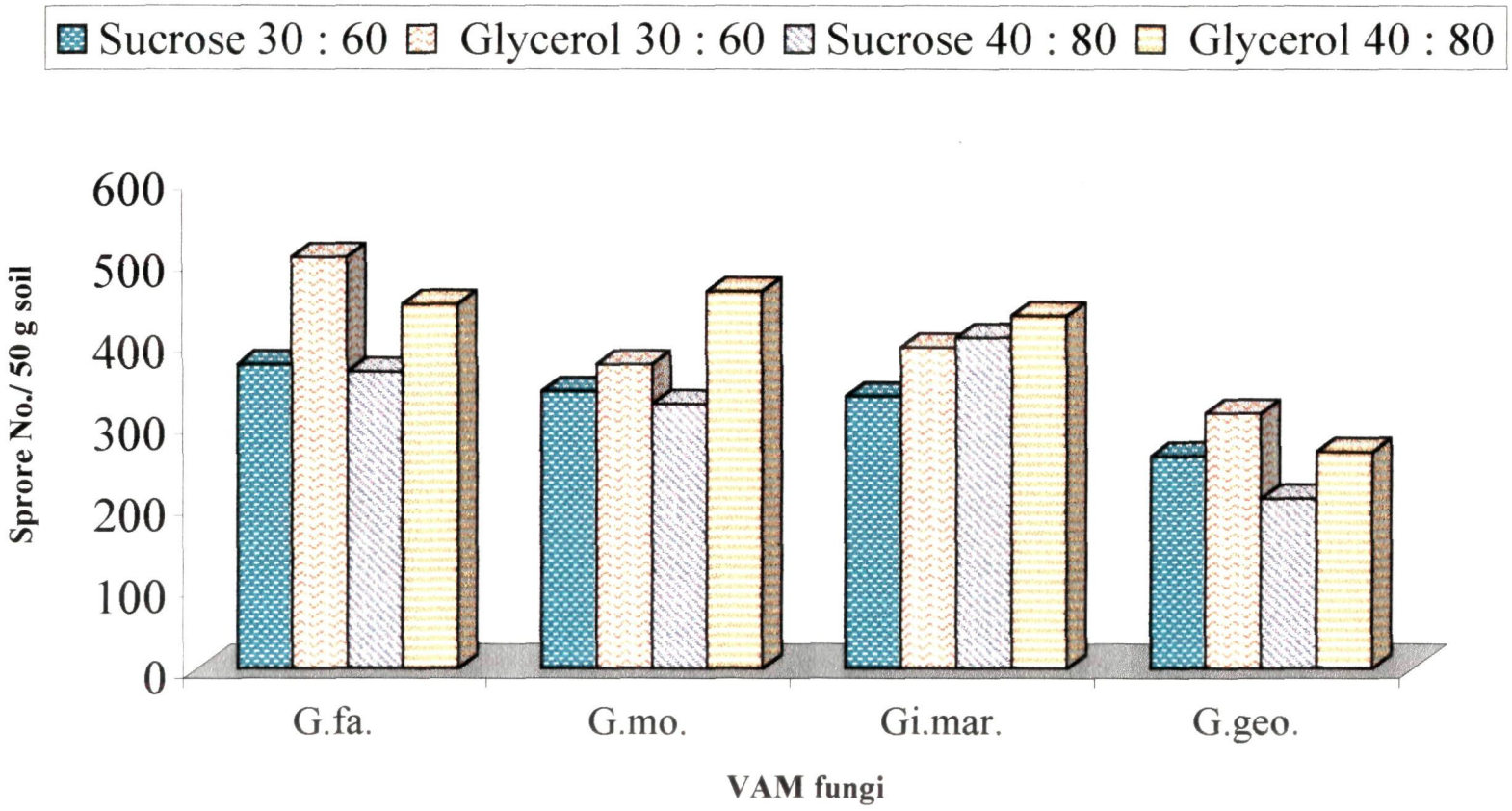
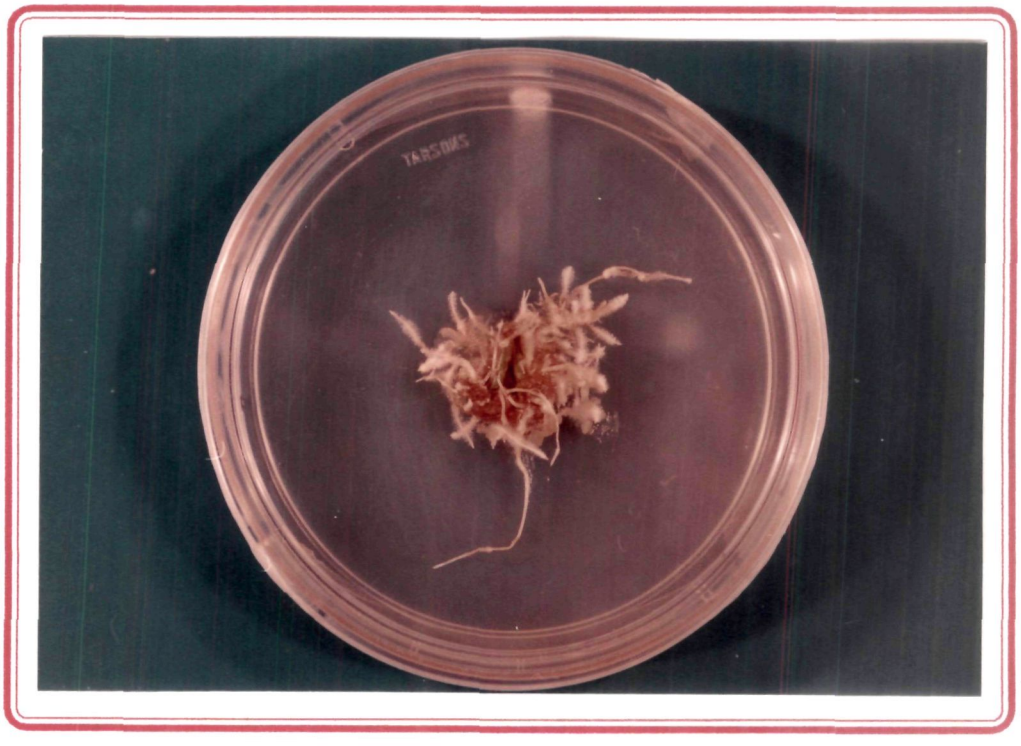


Fig. 15 : Retrieval of VAM spores by sucrose and glycerol gradient methods

A



B

PLATE 11: Effect of grwoth regulator on transformed roots

A. Normal root-without GR
B. Short and retorded growth with GR

fasciculatum spores recovered was 373 at 30 : 60 and 363 at 40 : 80 sucrose gradients. The number of spores recovered using glycerol gradient was 505 at 30 : 60 and 447 at 40 : 80 gradients.

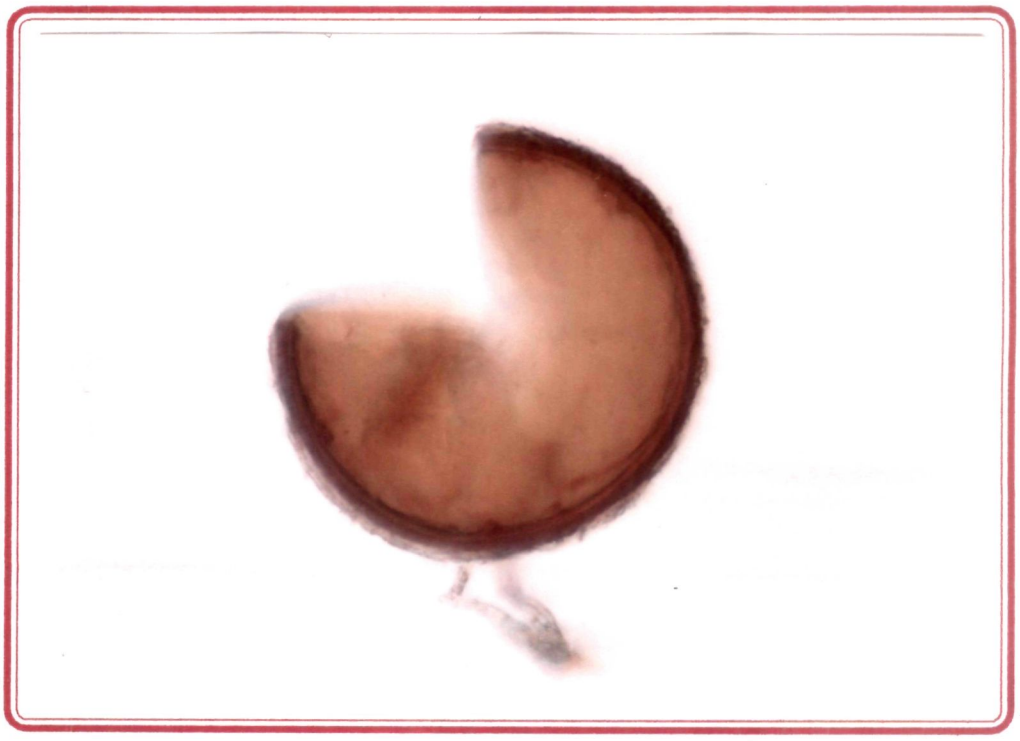
The recovery of *G. mosseae* by glycerol gradient of 40:80 was 463 and by 30:60 gradient it was 373. The number of spores isolated using sucrose gradient of 30:60 was 340 and with gradient of 40:80 it was 324. The recovery of *Gigaspora margarita* spores was also high in glycerol gradients compared to sucrose gradient. A similar result was also obtained in *G. geosporum*.

Isolation and identification of the predominant VAM fungus occurring in mine spoil :

The VAM spores isolated from mine spoils were purified and identified with the help of standard manual for identification (Schenck and Perez, 1988). The description of single predominant spore is given below. Sporocarps absent, chlamydosopores single in soil, globose to subglobose in shape ; the diameter 113 to 134 µm at maturity, spore surface smooth with dull appearance. The young spores were yellow to brown in colour, transparent to translucent ; the matured spores being dark yellow brown (Plate 12).

Spore wall 10.31 µm ; spore with one single funnel shaped subtending hypha (16 µm in diameter) with a hyphal constriction was clearly seen. Spore contents

A



B

PLATE 12: *Glomus geosporum* isolated from waste mine soil

were uniform oil droplets when young and becoming more granular when aged (Plate 13).

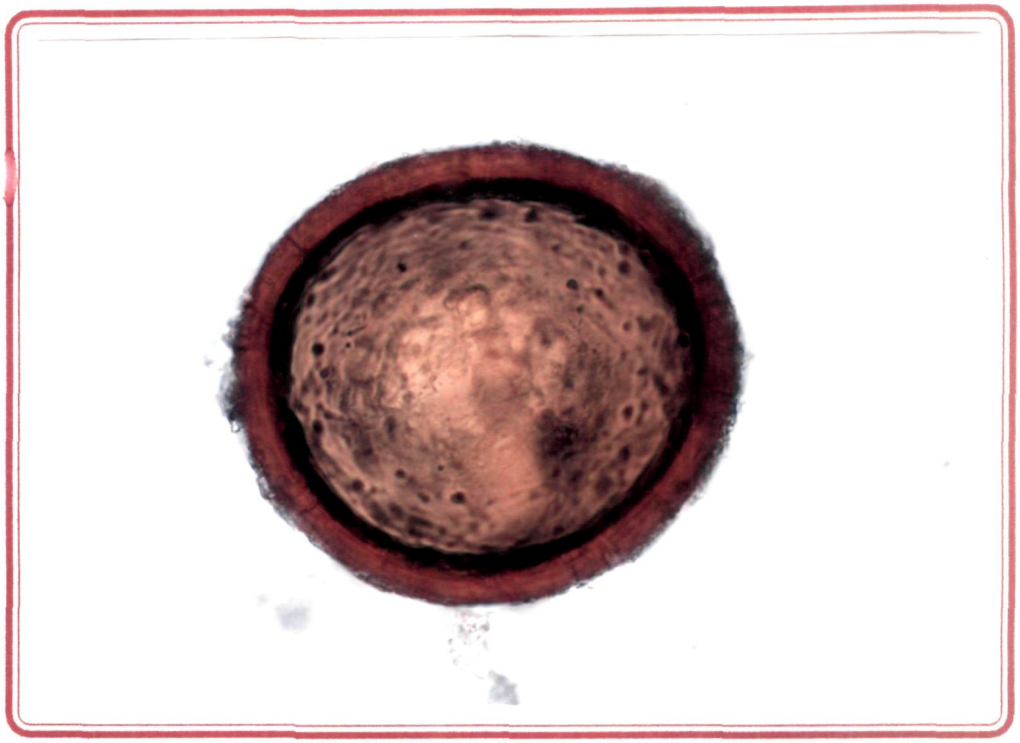
Based on the above characters the isolate was identified as *Glomus geosporum* Nicolson and Gerdemann.

VAM spore germination :

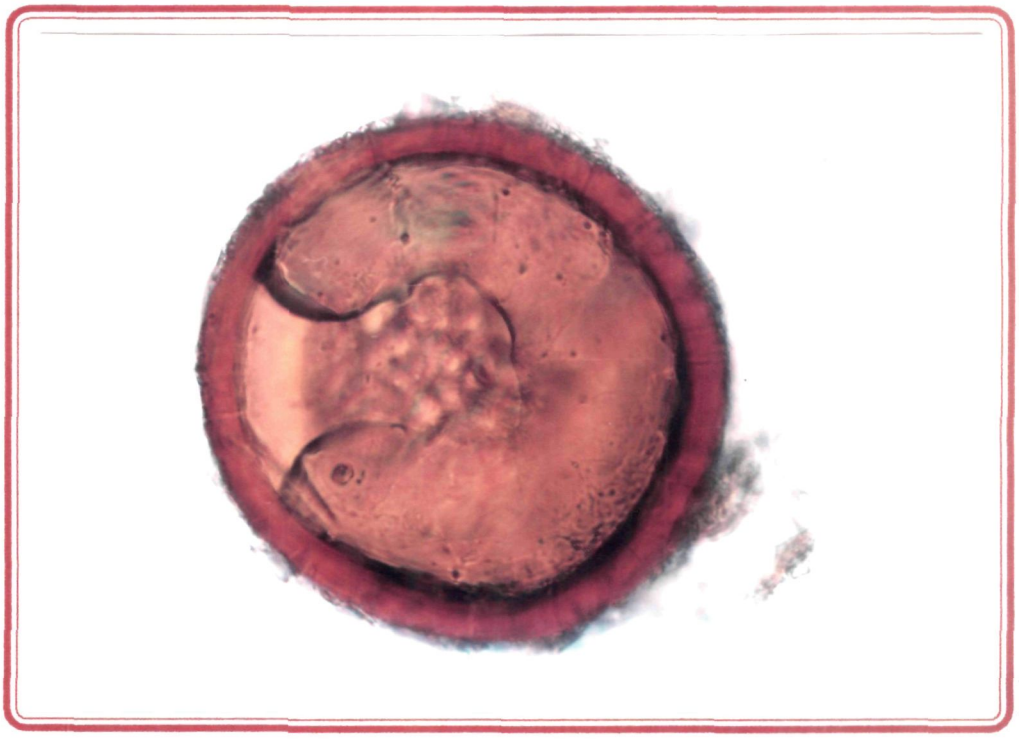
Spores of VAM fungi (*Glomus fasciculatum, Glomus mosseae, Gigaspora margarita and Glomus geosporum*) were sterilized by methods described earlier and were stored at 4^0 C. The sterilized spores were then transferred on water agar with sterile injection needles. To ensure efficient colonization with transformed roots pre-germinated VAM spores were used. Germination of VAM spores on water agar is shown in plate 14. An extensive hyphal development on water agar was noticed in *Glomus fasciculatum* (Plate 15).

Effect of crude cellulase enzyme on germination of VAM spores :

The chlamydospores of VAM fungi are mostly thick walled. This protects the spores and helps to tide over adverse conditions. Effect of cellulase enzyme may hasten the process of spore germination. To find out this effect, the experiment was laid out with different concentrations of crude cellulase in water agar to know its effect on VAM spore germination.

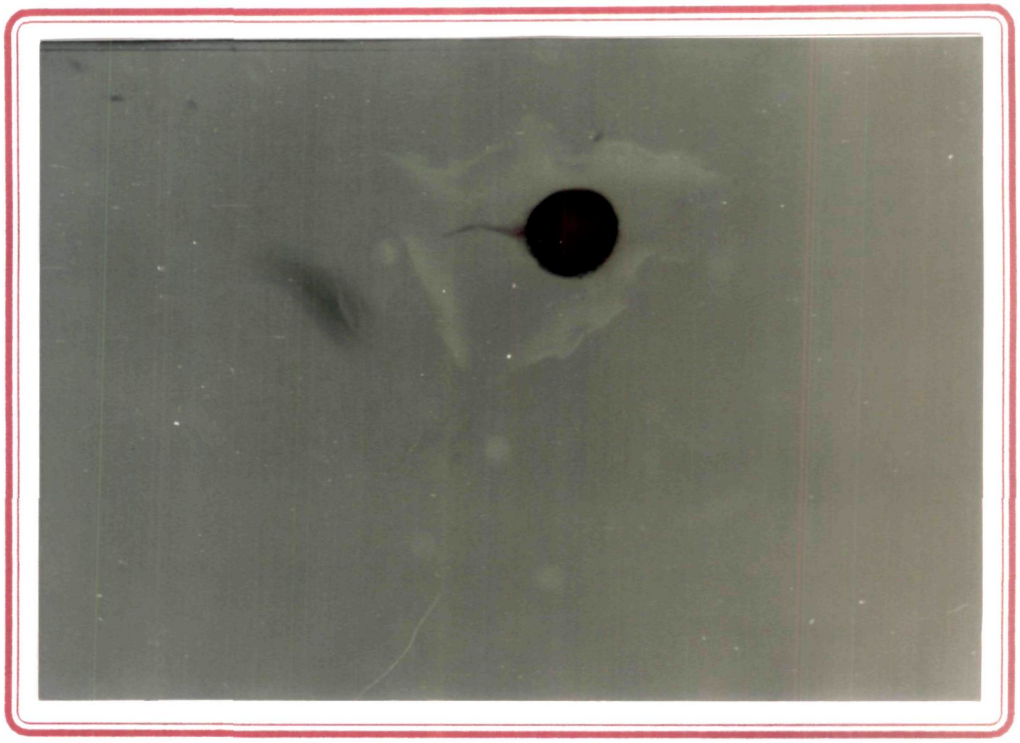


A

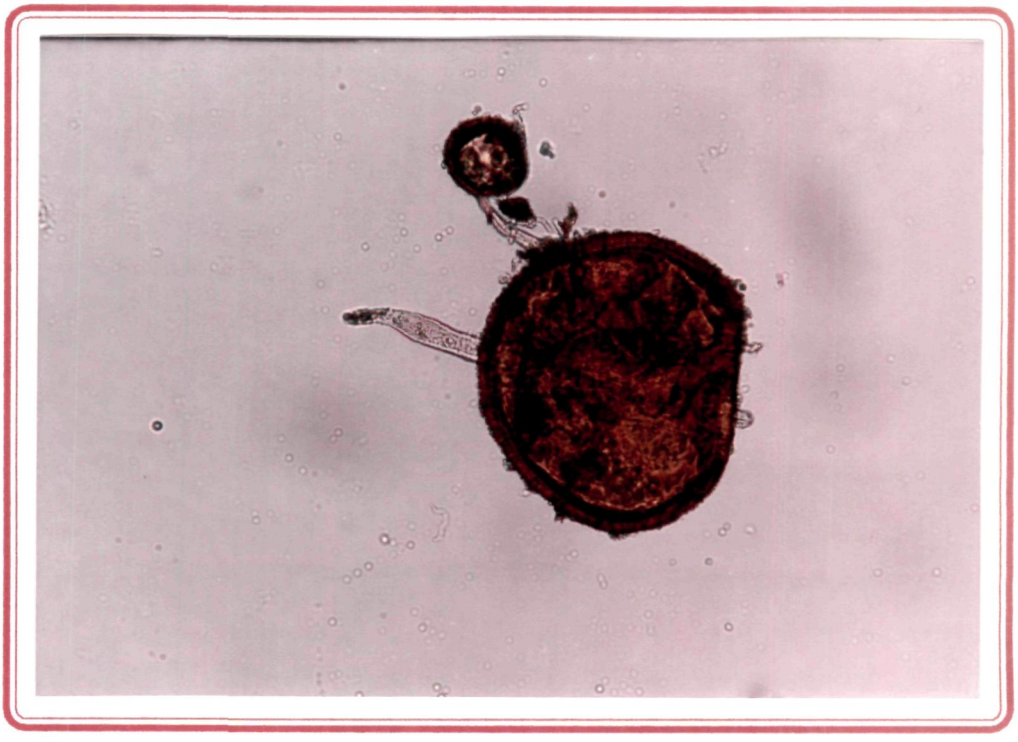


B

PLATE 13: Young (A) and matured (B) spores of *Glomus geosporum*

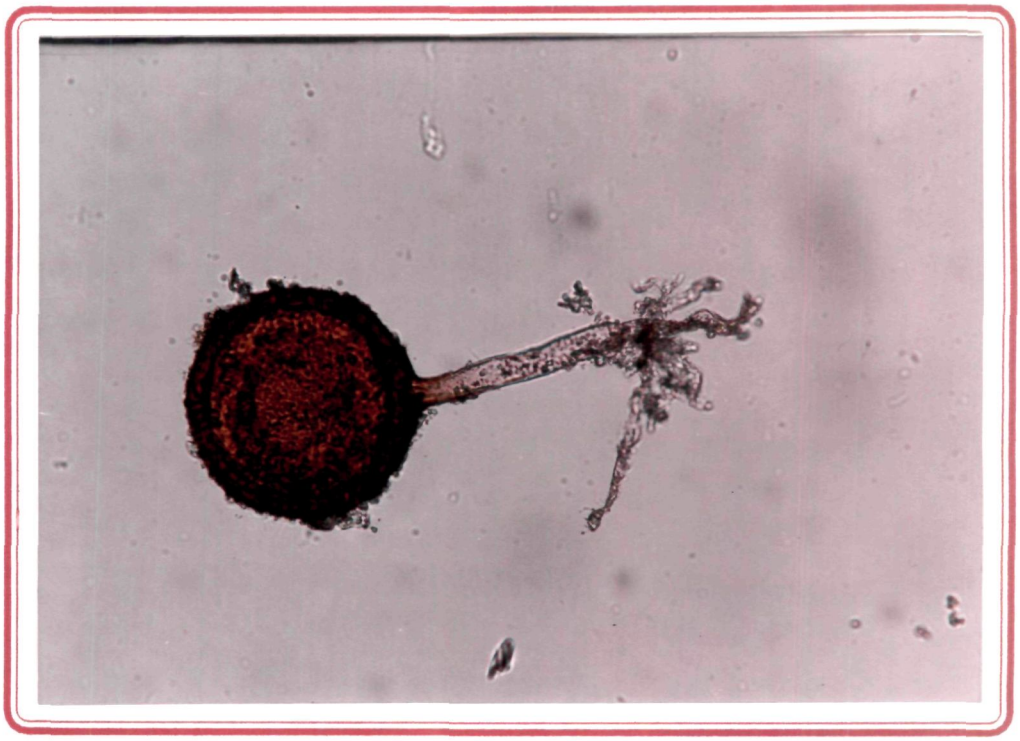


A

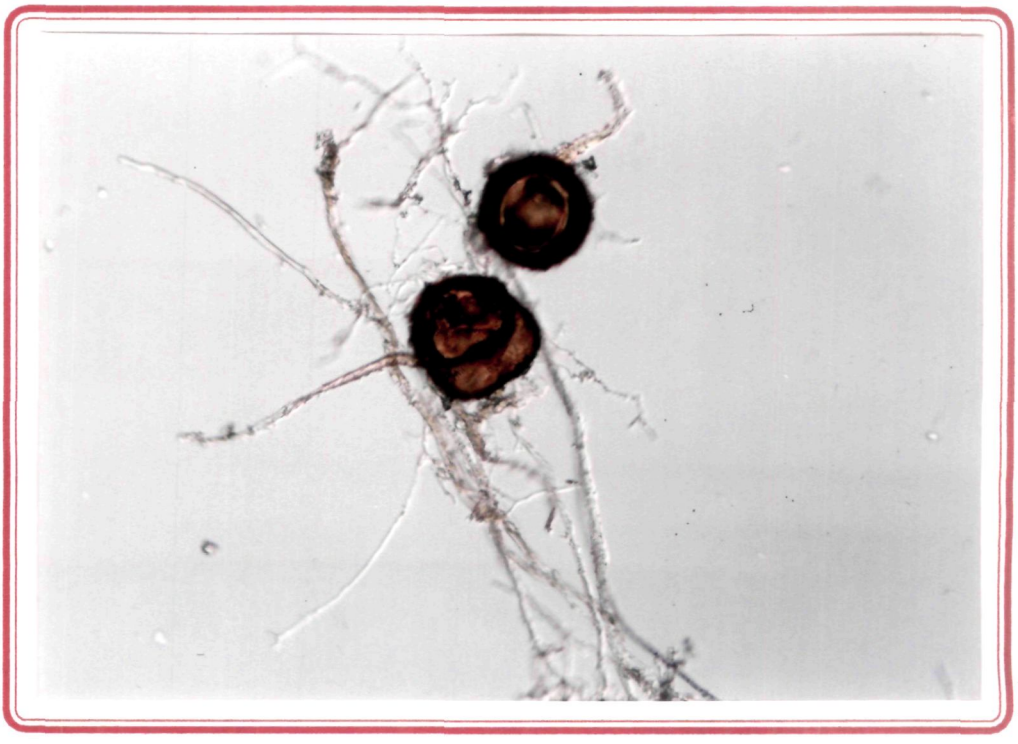


B

PLATE 14: Germination of *Gigaspora margarita* (A) and *Glomus geosporum* (B) on water agar



A



B

PLATE 15: Spore germination of *Glomus geosporum* (A) and hyphal development of *Glomus fasciculatum* (B) on water agar

The percentage germination of *G. fasciculatum* spores was the highest when the enzyme concentration used was 16 mg/l of enzyme (Table 20 and Fig.16). When 4 mg/l concentration was used the germination percentage was on par with untreated control. Use of 16 mg/l of crude cellulase also resulted in maximum spore germination of *G. mosseae*. Incorporation of 4 and 8 mg/l of enzyme in the medium did not significantly help spore germination compared to control. Higher concentration of enzyme (20 mg/l) reduced spore germination compared to control. More or less a similar trend was seen in *Gi. margarita*. *Glomus geosporum* responded well to enzyme treatment at 16 mg/l concentration (49.3%) followed by 8 mg/l (38.6%). All the other treatments, were statistically on par with each other (with a range of 29.0 to 33.3%).

Effect of bacterial and fungal cell-free extracts on germination of VAM spores :

Some of the soil microorganisms called mycorrhization helper bacteria (MHB) as well as some fungi stimulate mycorrhizal development.

Effect of bacterial cell-free extracts on spore germination :

The effect of sterilized culture cell-free extracts of beneficial bacteria on the germination of VAM spores is presented in Table 21. The mean percentage spore germination in control plants was 19.16. The cell-free culture filtrate of *Azospirillum brasilense* added to the water agar at 10 per cent concentration resulted in maximum germination of VAM spores (30.80%). This was followed by the

Table 20 : Effect of different concentrations of cellulase on spore germination of VAM fungi

Cellulase concentration (mg/l)	Spore germination (%)				
	G. fa.	*G. mo.*	*Gi. mar.*	*G. geo.*	Mean
4 .00	36.00	38.60	37.30	29.30	35.30
8 .00	30.60	36.00	46.60	38.60	37.75
12 .00	30.60	33.30	34.60	34.60	33.27
16 .00	45.30	53.30	56.00	49.30	50.97
20 .00	26.60	20.00	28.00	30.60	26.30
Control (without enzyme)	38.60	37.30	41.30	33.30	37.62
Mean	8.66	9.11	10.16	9.11	

	SEM ±	CD (P=0.05)
VAM fungi	0.58	1.67
Cellulase concentration	0.71	2.04
Interaction	1.43	4.09
CV %	28.89	

G. fa - *Glomus fasciculatum*
G. mo - *Glomus mosseae*
Gi. mar - *Gigaspora margarita*
G. geo - *Glomus geosporum*

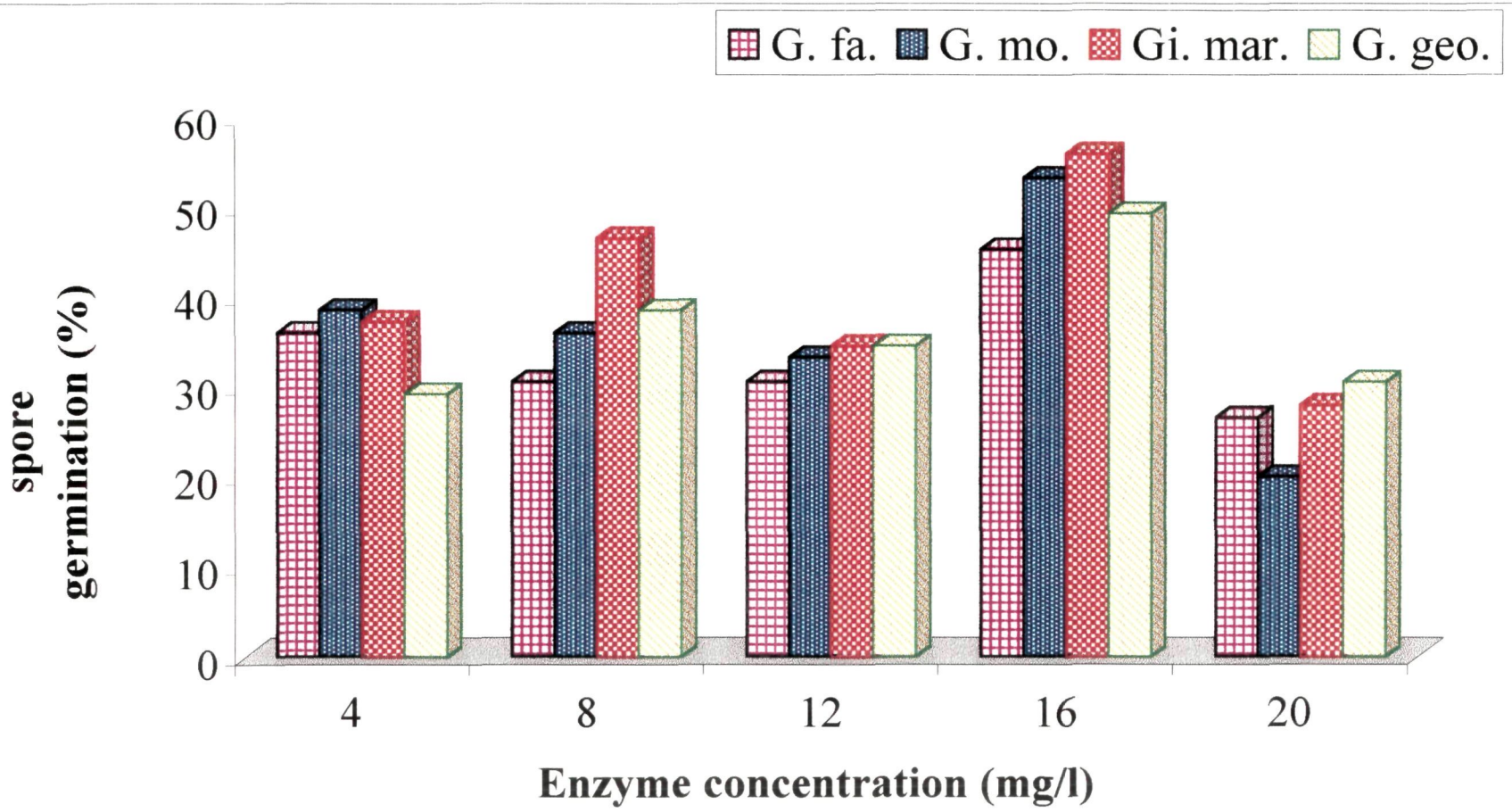


Fig. 16 : Effect of different concentrations of cellulase on spore germination of VAM fungi

Table 21 : Effect of different concentrations of bacterial cell-free extracts on spore germination of VAM fungi

Bacterial cell-free extracts	Spore germination (%)				
	G. fa.	*G. mo.*	*Gi. mar.*	*G. geo.*	Mean
Control	20.00	23.30	13.30	20.00	19.16
Bacillus megatherium - 2.5%	16.66	20.00	16.66	20.00	18.30
5.0%	13.33	16.66	20.00	20.00	17.50
10.0%	16.60	20.33	16.00	10.00	15.80
Bacillus coagulans - 2.5%	13.33	16.66	16.66	13.33	15.00
5.0%	23.30	23.30	23.30	30.00	25.00
10.0%	23.30	16.60	23.30	23.30	21.60
Azospirillum brasilense - 2.5%	16.60	13.30	23.30	23.30	19.16
5.0%	23.30	20.00	26.60	23.30	23.30
10.0%	30.00	23.00	33.30	36.60	30.80
Azotobacter chroococcum - 2.5%	16.60	20.00	20.00	26.60	20.80
5.0%	20.00	23.00	23.30	30.00	24.16
10.0%	23.30	26.60	23.30	30.00	25.80
Rhizobium sp. - 2.5%	16.60	20.00	16.60	20.00	18.30
5.0%	23.30	13.30	23.30	26.00	21.60
10.0%	20.00	26.60	20.00	16.60	20.80
Mean	19.81	20.37	20.93	23.15	

	SEM ±	CD (P=0.05)
Cell-free extracts	0.15	0.43
Concentrations	0.11	0.30
VAM fungi	0.12	0.35
CV %	44.29	

G. fa – *Glomus fasciculatum*
G. mo – *Glomus mosseae*
Gi. mar – *Gigaspora margarita*
G. geo – *Glomus geosporum*

addition of *Azotobacter chroococcum* filtrate at 10 per cent concentration resulting in 25.80% spore germination. Culture filtrate of *Bacillus megatherium* reduced the VAM spore germination compared to control at all the three concentrations studied. Addition of the culture filtrate of *B. coagulans* and *Rhizobium* sp. at 5 and 10 per cent concentration enhanced VAM spore germination while its addition at 2.5 per cent level reduced spore germination.

Effect of fungal cell-free extracts on spore germination :

Effect of culture filtrates of two plant growth promoting fungi (*Trichoderma harzianum* and *Aspergillus awamori*) and three cellulose decomposing fungi *Aspergillus* sp. LC_4, *Trichoderma* sp. LC_5 and *Phenocheate chrysosporium* on VAM spore germination is given in Table 22. The mean percentage germination of VAM spore in control plates was 15.21. Addition of the cell-free extract of *Trichoderma* sp. (LC_5) significantly enhanced spore germination to the maximum at all the concentrations studied, while addition of the cell-free extract of *Aspergillus* sp. (LC_4) had the opposite effect reducing spore germination at two concentrations studied compared to control. Addition of the culture filtrate of *Trichoderma harzianum* and *Aspergillus awamori* did not have much influence on spore germination compared to control. Culture filtrate of *Phenocheate chrysosporium* significantly enhanced spore germination at all concentrations, though not to the extent of *Trichoderma* sp. (LC_5).

Table 22 : Effect of different concentrations of fungal cell-free extracts on spore germination of VAM fungi

Fungal cell-free extracts		Spore germination (%)				
		G. fa.	*G. mo.*	*Gi. mar.*	*G. geo.*	Mean
Contorl		15.59	16.55	14.62	17.74	16.12
Trichoderma harzianum	- 2.5%	16.77	14.40	16.55	14.62	15.58
	5.0%	14.62	14.62	13.86	16.77	14.97
	10.0%	16.77	15.58	11.71	14.62	14.67
Aspergillus awamori	- 2.5%	14.62	12.67	14.62	14.62	14.13
	5.0%	12.89	14.62	16.77	15.81	15.02
	10.0%	15.80	14.40	13.44	14.62	14.56
Aspergillus sp. (LC_4)	- 2.5%	13.44	8.80	11.71	13.44	11.84
	5.0%	14.62	11.71	16.77	17.42	15.21
	10.0%	14.62	13.43	14.40	13.44	13.97
Trichoderma sp. (LC_5)	- 2.5%	20.29	20.84	20.29	20.97	20.59
	5.0%	22.70	24.07	26.04	24.81	24.40
	10.0%	20.97	23.38	22.55	22.55	22.36
Phenocheate chrysosporium	- 2.5%	15.58	14.62	14.62	16.77	15.40
	5.0%	13.44	15.58	16.55	17.74	15.83
	10.0%	14.62	15.58	14.40	17.37	15.50
Mean		15.94	15.55	16.12	17.05	

	SEM ±	CD (P=0.05)
Cell-free extracts	0.04	0.13
Concentrations	0.03	0.09
VAM fungi	0.04	0.11
CV %	18.35	

G. fa – *Glomus fasciculatum*
G. mo – *Glomus mosseae*
Gi. mar – *Gigaspora margarita*
G. geo – *Glomus geosporum*

Co-culturing VAM in transformed roots :

Surface sterilized VAM spores (*Glomus fasciculatum, Glomus mosseae, Gigaspora margarita and Glomus geosporum*) were incubated for spore germination. Pre-germinated spores were spotted aseptically on transformed tomato roots 1 to 2 cm above the growing tips. These plates were incubated vertically to ensure infection. The germinating spores infected and penetrated the host (Plate 16).

The microscopic observation of the roots after 12 to 15 days showed colonization by VAM fungi resulting in extensive extramatricular hyphae (Plate 17). The intraradical mycelial development when studied gave the root a beaded appearance (Plate 18a). Arbuscules were seen after 15 to 18 days of incubation (Plate 19). Examination after 20 to 22 days revealed the presence of vesicles in addition to the arbuscules (Plate 18b).

VAM colonization in transformed roots :

Percentage VAM colonization in transformed roots incubated at 25^0C is presented in Table 23 and Fig.17. In *A. rhizogenes* strain LBA – 9402 transformed tomato roots percentage colonization was maximum when co-cultured with *G. margarita* (31.78%) followed by roots co-cultured with *G. fasciculatum* (27.15%). The percentage colonization of transformed roots by *G. mosseae* and

Table 23 : VAM colonization in transformed tomato hairy roots induced by different strains of *Agrobacterium rhizogenes* at 25^0 C

VAM fungi	Colonization (%)			
	Agrobacterium rhizogenes			
	LBA-9402	**ATCC-15834**	**A_4**	**Mean**
1. *Gigaspora margarita*	31.78	26.26	27.53	28.52
2. *Glomus fasciculatum*	27.15	20.53	24.49	24.05
3. *Glomus mosseae*	22.20	21.06	21.54	21.60
4. *Glomus geosporum*	26.46	19.16	20.08	21.90
Mean	26.89	21.75	23.41	

	SEM ±	**CD (P=0.05)**
A. rhizogenes strains	1.33	3.79
VAM fungi	1.53	4.37
Interaction	2.66	7.58
CV %	24.80	

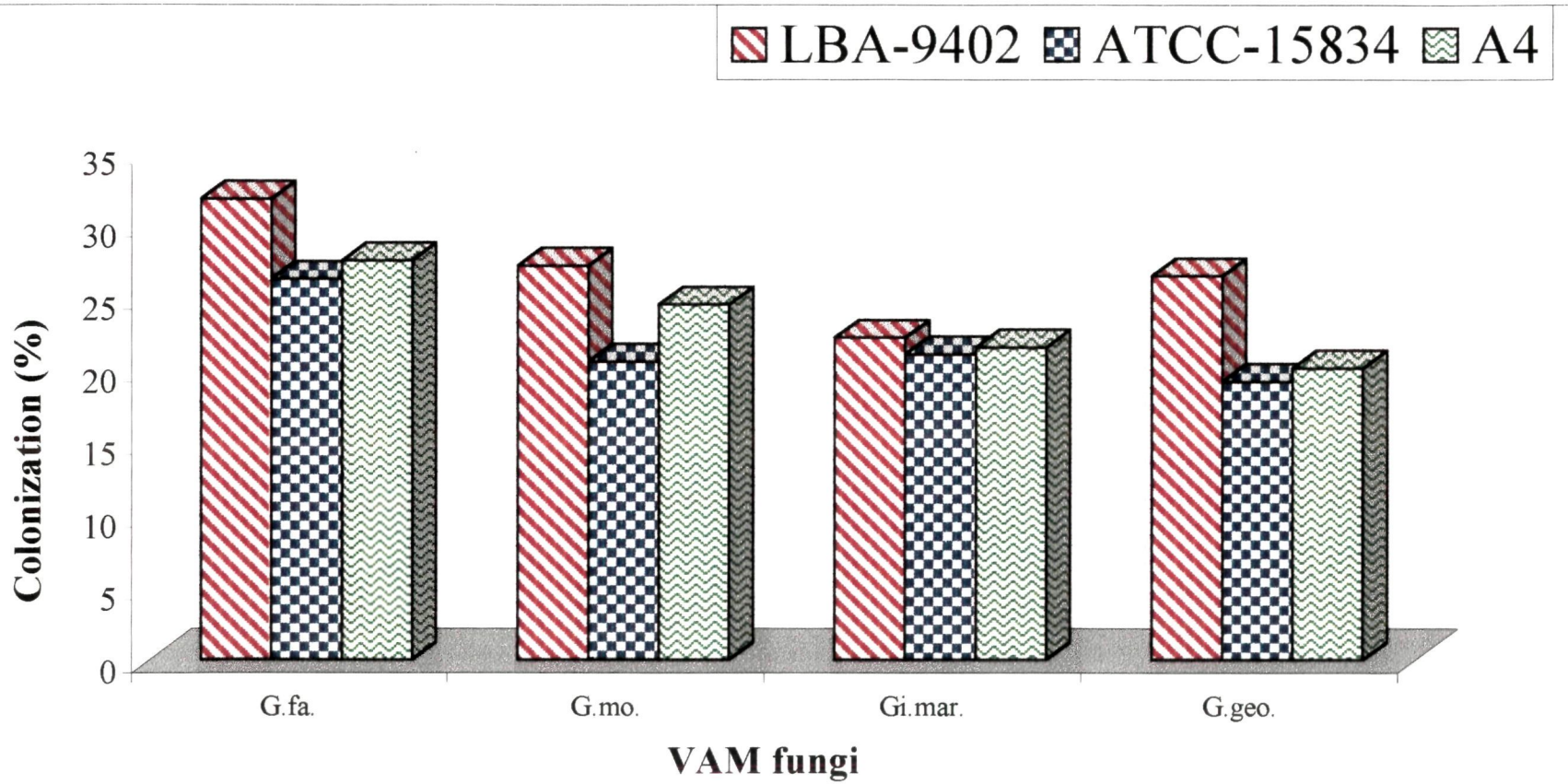
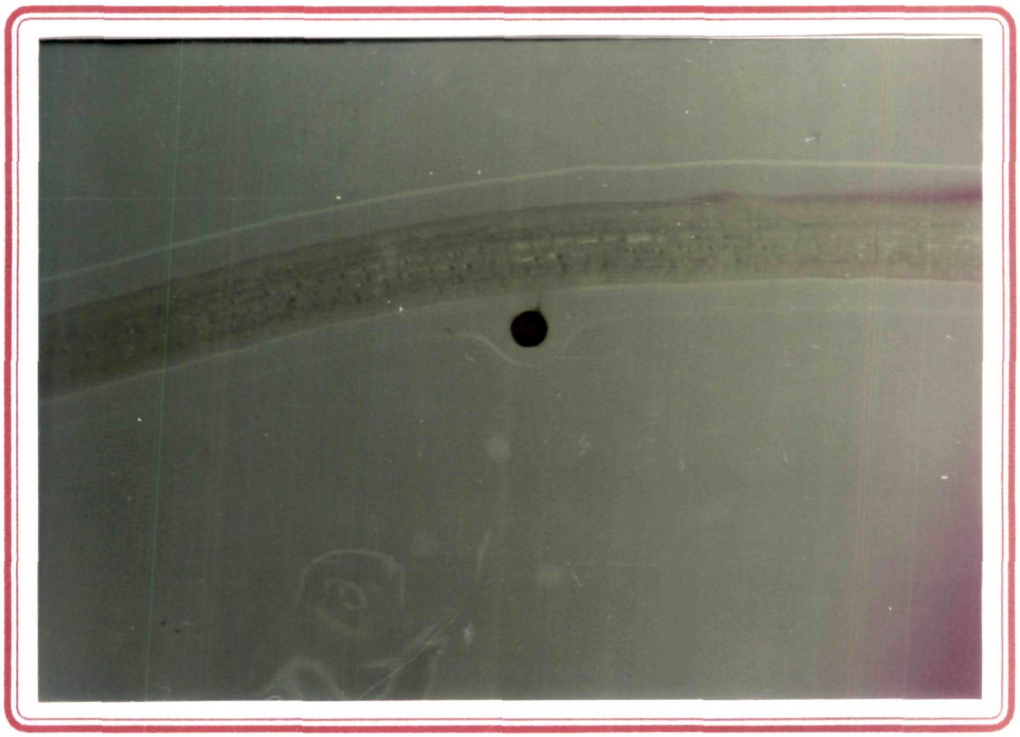


Fig. 17 : VAM colonization in transformed tomato hairy roots induced by different strains of *Agrobacterium rhizogenes* at 25^0C



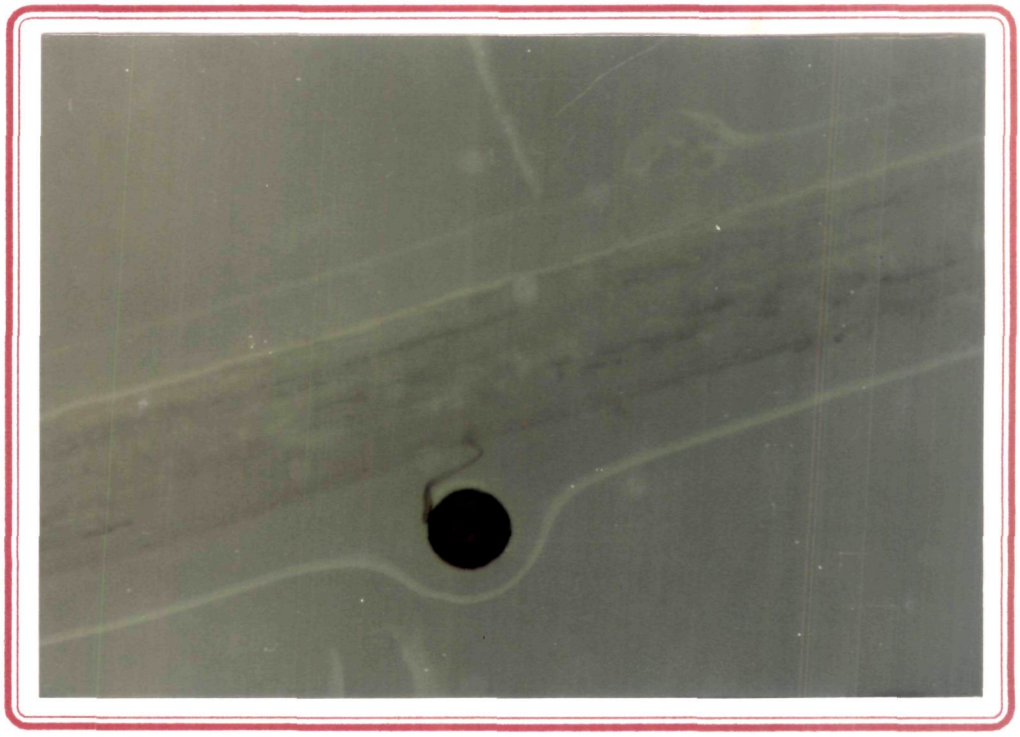
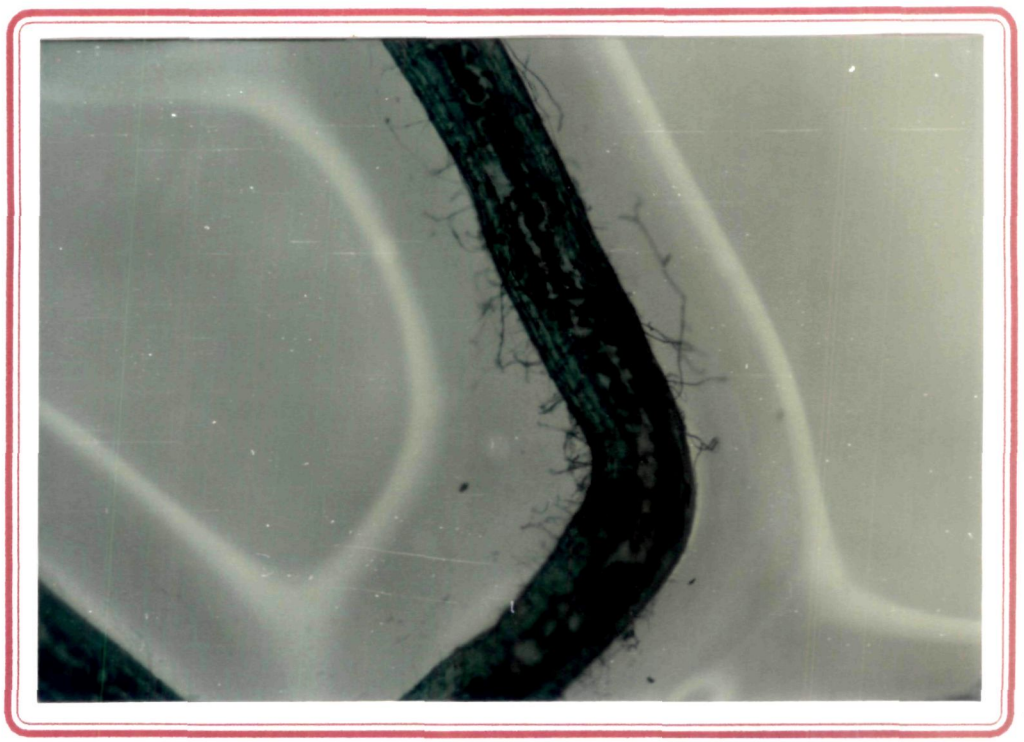
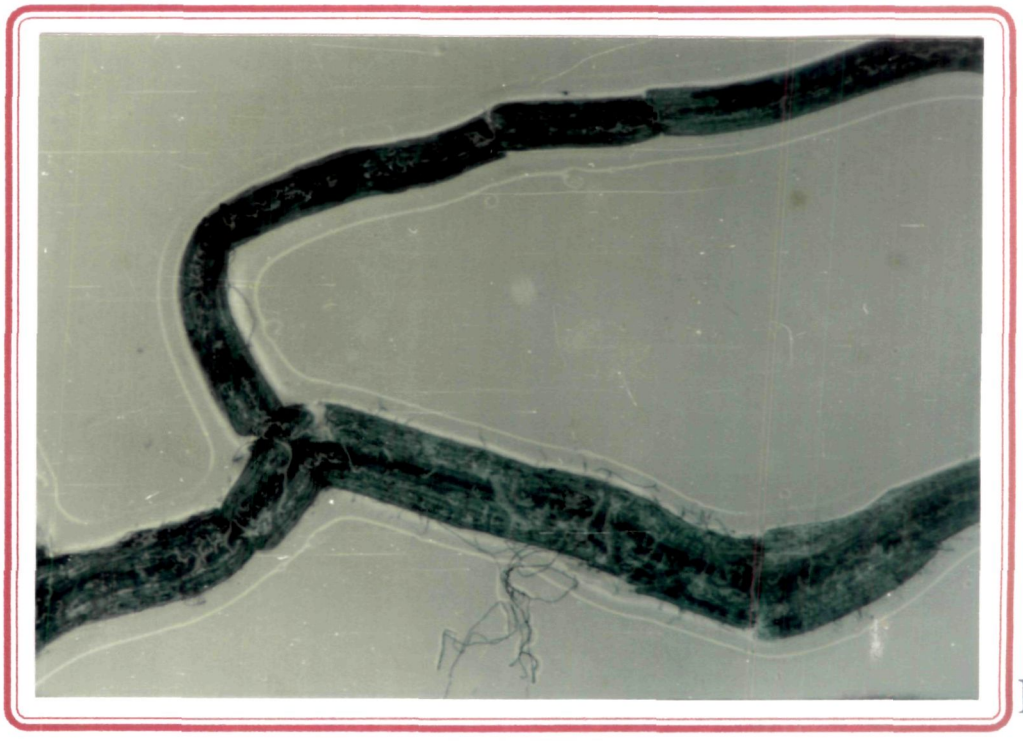


PLATE 16: Co-culturing of *Gigaspora margarita* with transformed tomato roots - spore germination and penetration of the root

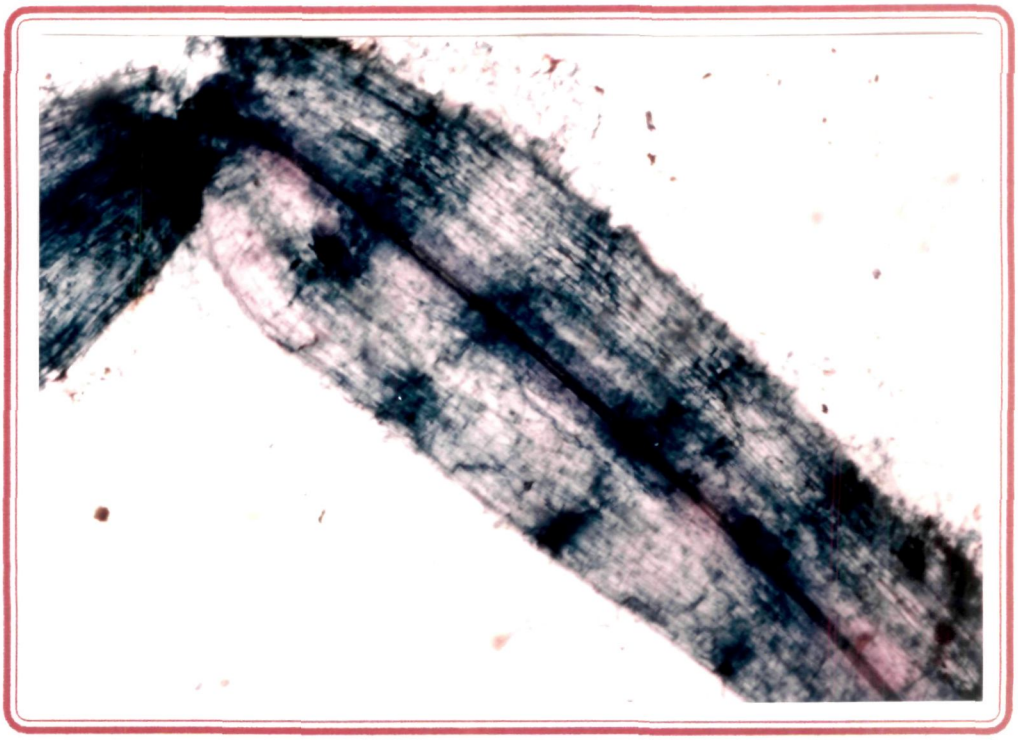


A



B

PLATE 17: Colonization transformed roots by *Gigaspora margarita* (A) and *Glomus geosporum* (B) showing extramatricular hyphae

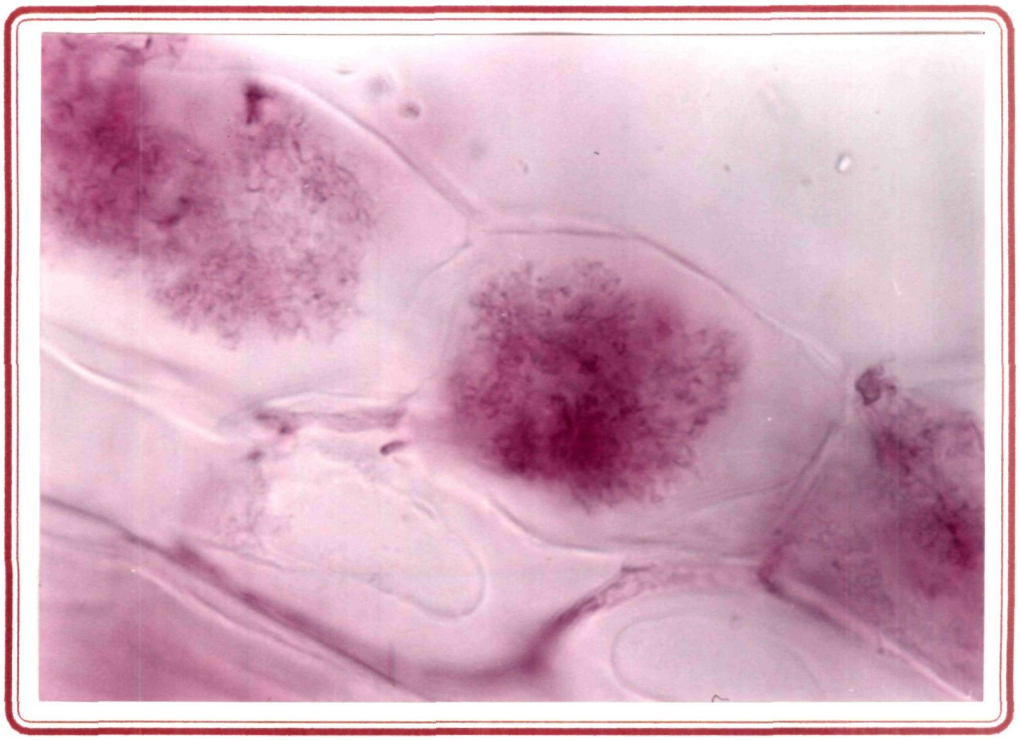


A

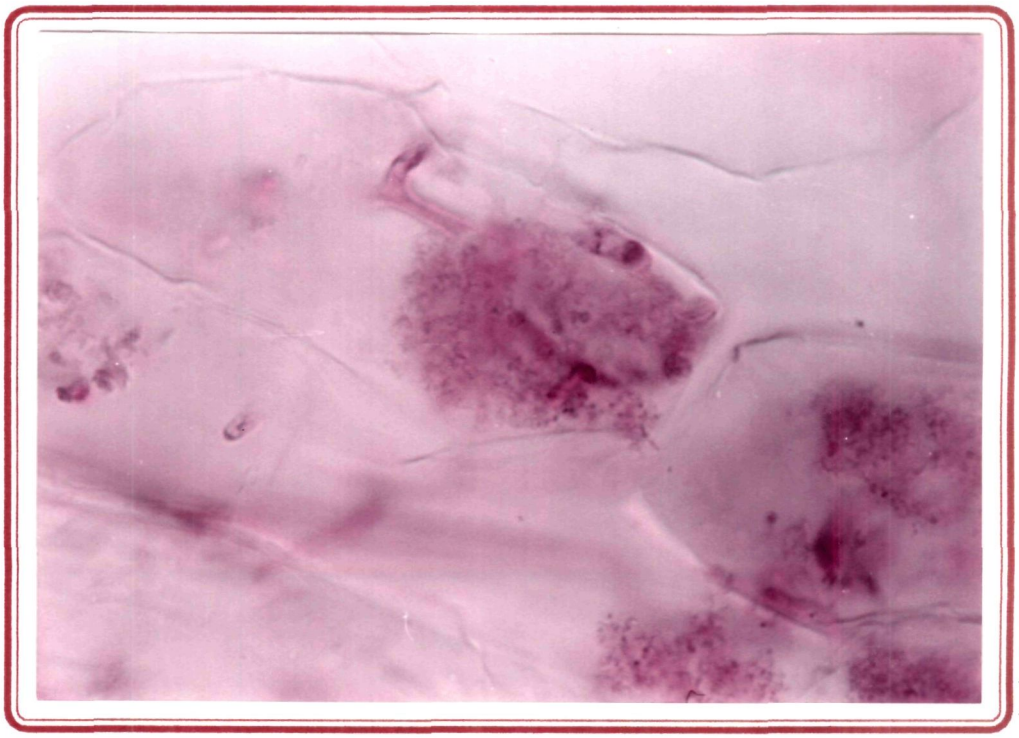
B

PLATE 18: Colonization transformed tomato roots by *Glomus geosporum*

A. Intraradical mycelial development
B. Arbuscules and vesicles formation



A



B

PLATE 19: Arbuscules inside transformed tomato roots by *Glomus geosporum* (A) and *Gigaspora margarita* (B)

Glomus geosporum were 22.20% and 26.46% respectively but statistically on par with each other.

The mean percentage mycorrhizal colonization of roots transformed by *A. rhizogenes* strains A_4 and ATCC – 15834 were 23.41 and 21.75 respectively. Maximum colonization was observed in hairy roots induced by *A. rhizogenes* strain LBA – 9402. Of the four VAM fungi studied maximum root colonization was produced by *Gigaspora margarita* (28.52%), followed by *G. fasciculatum* (24.05%). *G. mosseae* and *G. geosporum* produced 21.60 and 21.90 per cent colonization respectively.

The per cent colonization by VAM fungi in transformed roots incubated at 30^0C is presented in Table 24 and Fig.18. Maximum colonization of 33.50% was recorded in *A. rhizogenes* strains LBA – 9402 transformed tomato roots when co-cultured with *Gigaspora margarita* and this was followed by roots co-cultured with *Glomus geosporum* (25.53%). The percentage colonization of the transformed roots by *G. fasciculatum* and *G. mosseae* were 21.64 and 20.00 per cent respectively but statistically on par with each other.

The mean percentage of mycorrhizal colonization of roots transformed by ATCC – 15834 and A_4 were 18.61 and 23.28 per cent, respectively. Maximum colonization was observed in strain LBA – 9402. Among the four VAM fungi studied maximum was colonization was caused by *Gigaspora margarita* (27.66%)

Table 24 : VAM colonization in transformed tomato hairy roots induced by different strains of *Agrobacterium rhizogenes* at 30^0 C

VAM fungi	Colonization (%)			
	Agrobacterium rhizogenes			
	LBA-9402	ATCC-15834	A_4	Mean
1. *Gigaspora margarita*	33.50	22.24	27.25	27.66
2. *Glomus fasciculatum*	21.64	16.45	19.31	19.13
3. *Glomus mosseae*	20.00	17.88	21.18	19.69
4. *Glomus geosporum*	25.53	17.89	25.39	22.94
Mean	25.17	18.61	23.28	

	SEM ±	CD (P=0.05)
A. rhizogenes strains	1.24	3.54
VAM fungi	1.43	4.09
Interaction	2.48	7.08
CV %	24.89	

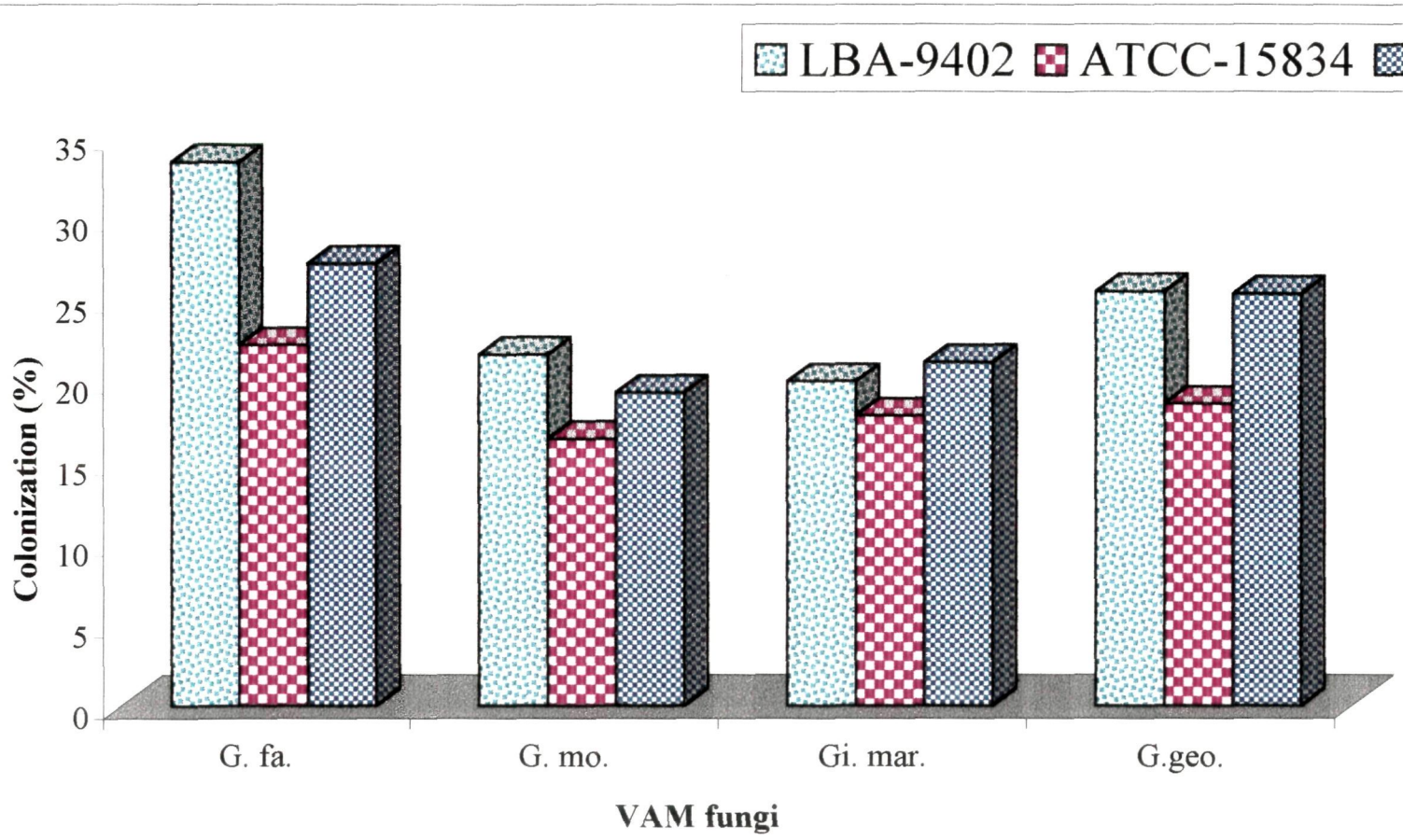
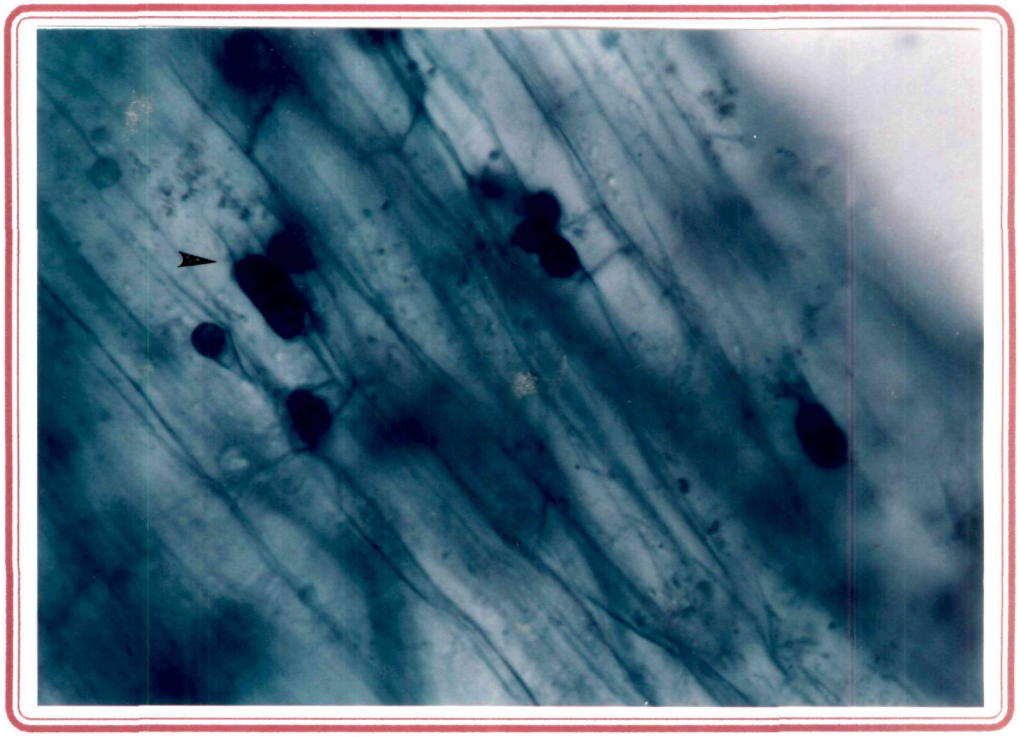


Fig. 18 : VAM colonization in transformed tomato hairy roots induced by different strains of *Agrobacterium rhizogenes* at 30^0 C.

followed by *Glomus geosporum* (22.94%). Both *G. fasciculatum* and *G. mosseae* produced almost same per cent colonization (19.13 and 19.69%).

The microscopic observations were made periodically on the transformed roots inoculated with four different VAM fungi, *Glomus fasciculatum, Glomus mosseae, Gigaspora margarita* and *Glomus geosporum* separately. Good colonization with abundant vesicles produced inside the root could be seen in all four co-cultures (Plates 20, 21 and 22). Extensive extramatricular mycelium also developed (Plate 23). Sporulation though occurred on the extramatricular mycelium after 35 to 40 days was sparse (Plates 24, 25 and 26).



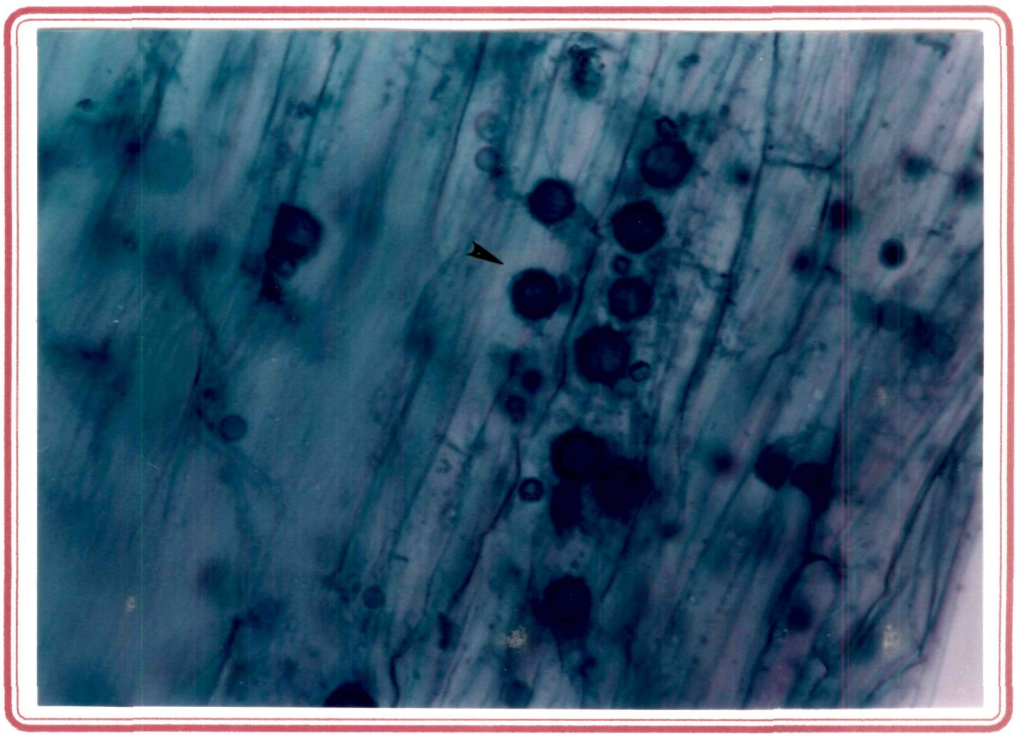
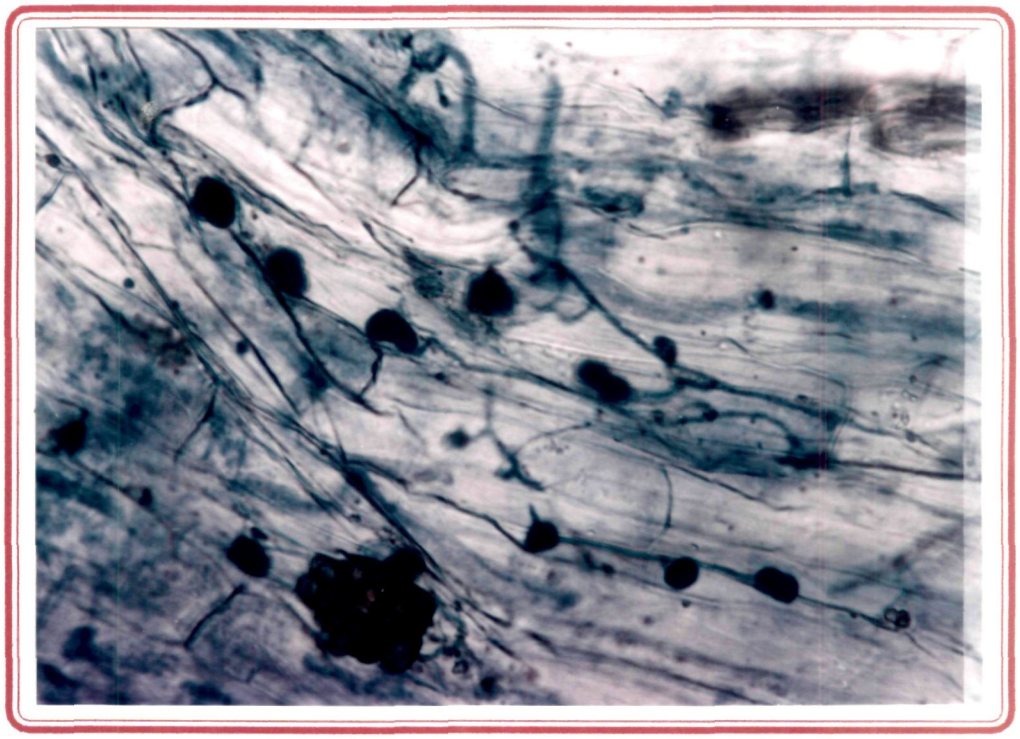
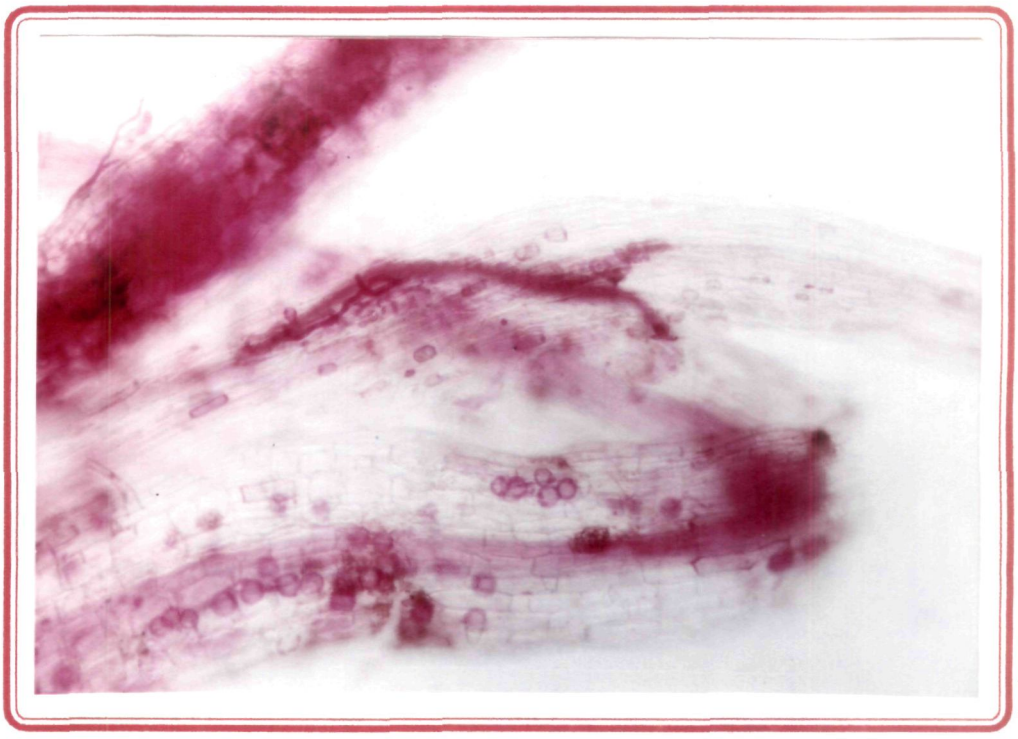


PLATE 20: Vesicles of *Glomus mosseae* inside the transformed tomato roots

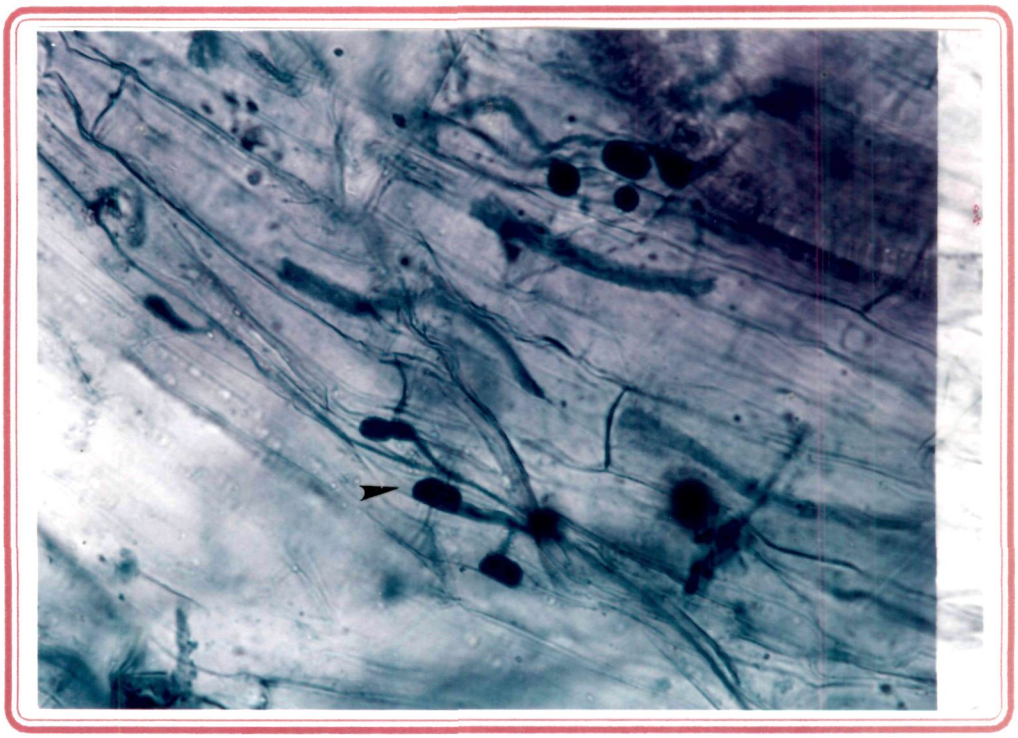


A



B

PLATE 21: Vesicles of *Glomus fasciculatum* (A) and *Glomus mosseae* (B) inside transformed tomato roots



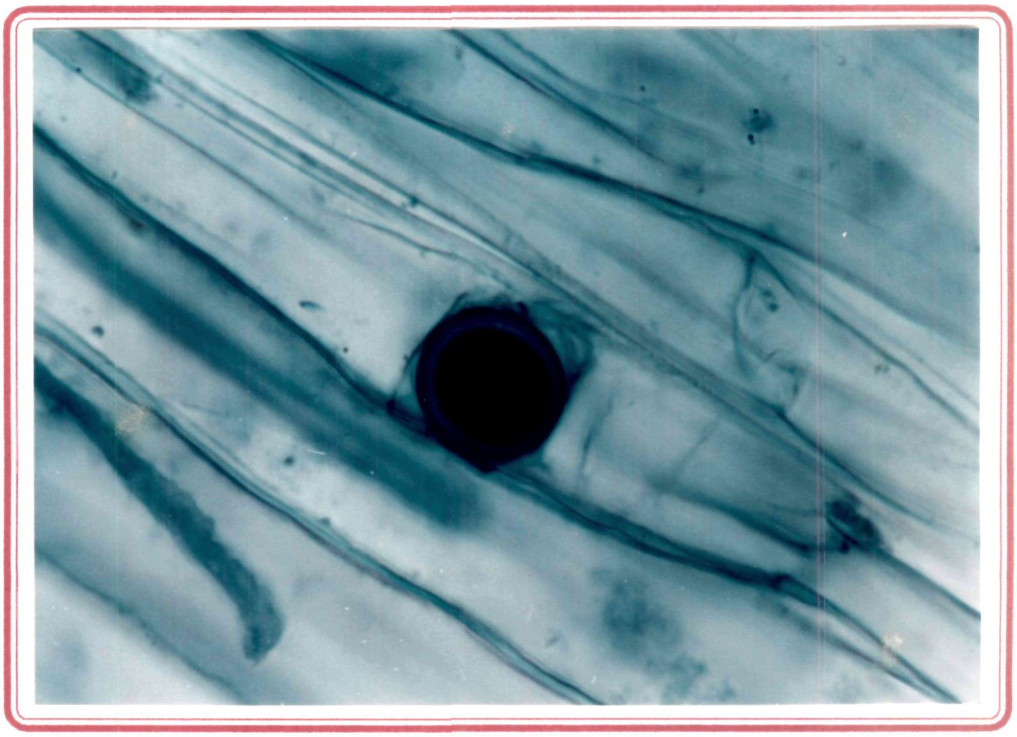
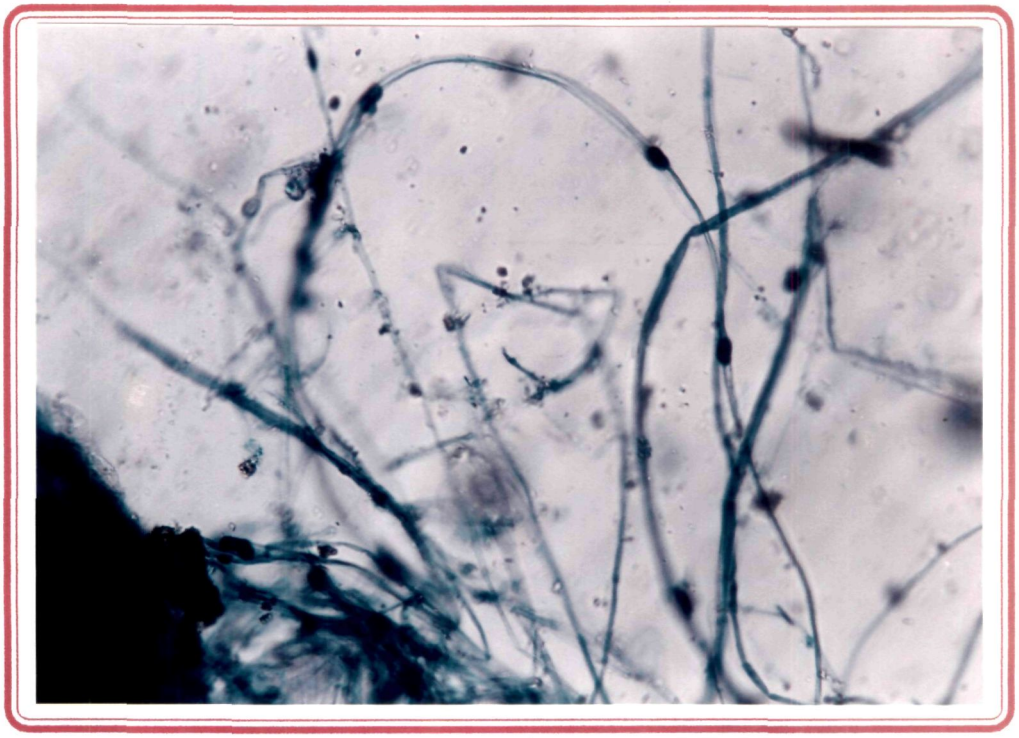
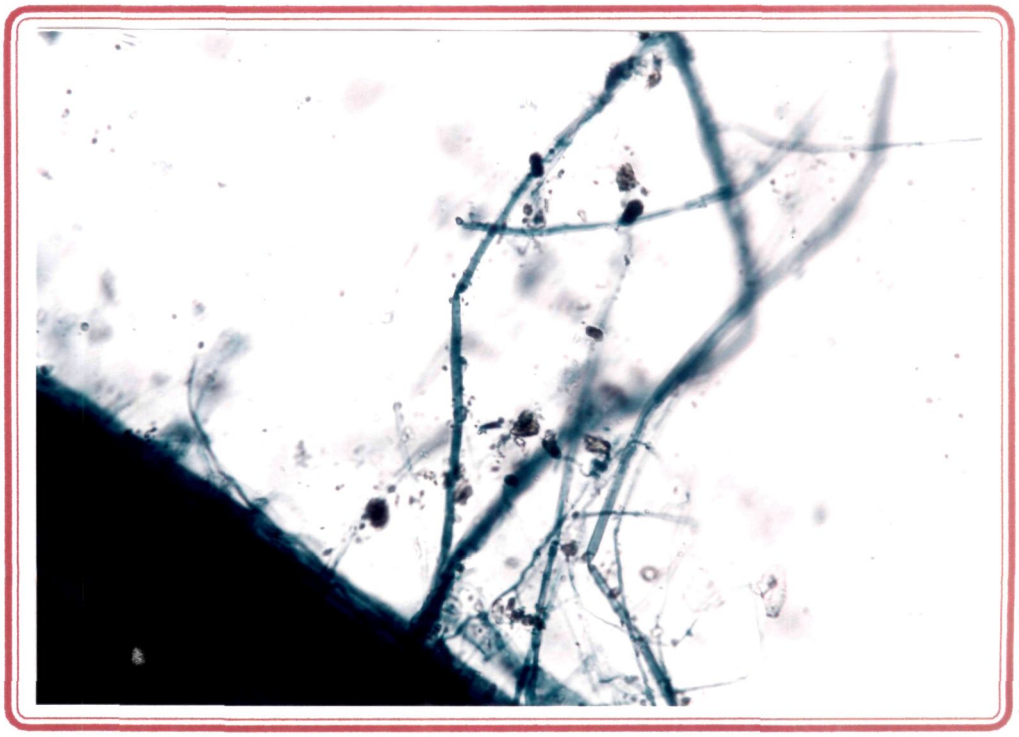


PLATE 22: Vesicles of *Glomus geosporum* inside transformed tomato roots

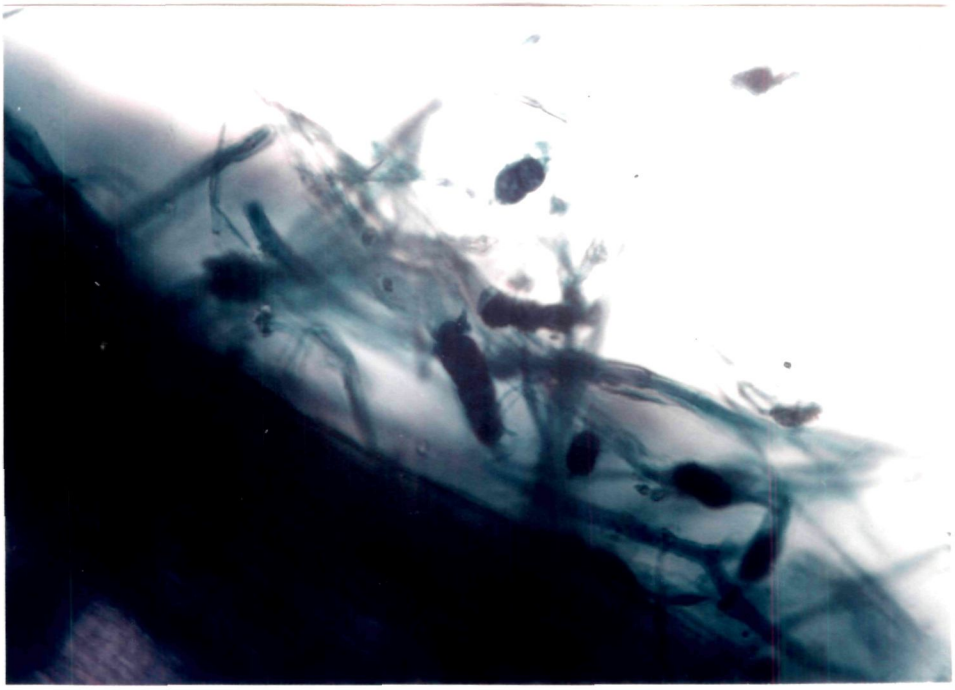


A



B

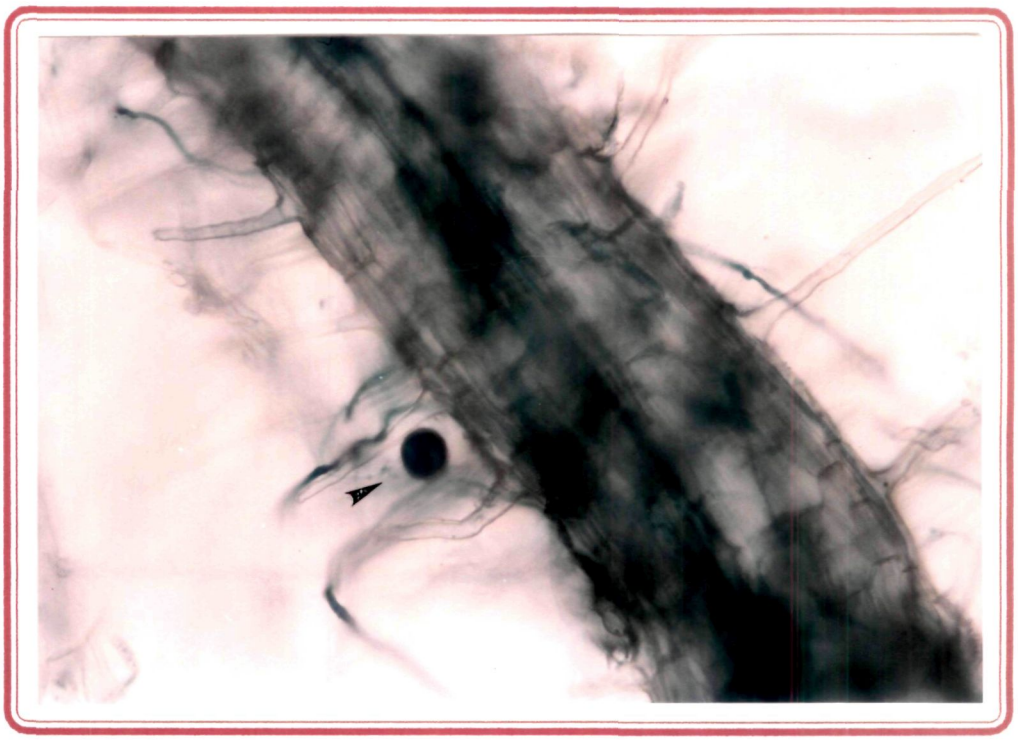
PLATE 23: Extramatricular mycelium of *Glomus geosporum* (A) and *Glomus fasciculatum* (B) from transformed tomato roots



A

B

PLATE 24: Sporulation of *Glomus fasciculatum* out side the root (A) and spores of *Glomus geosporum* inside the transformed tomato root (B)



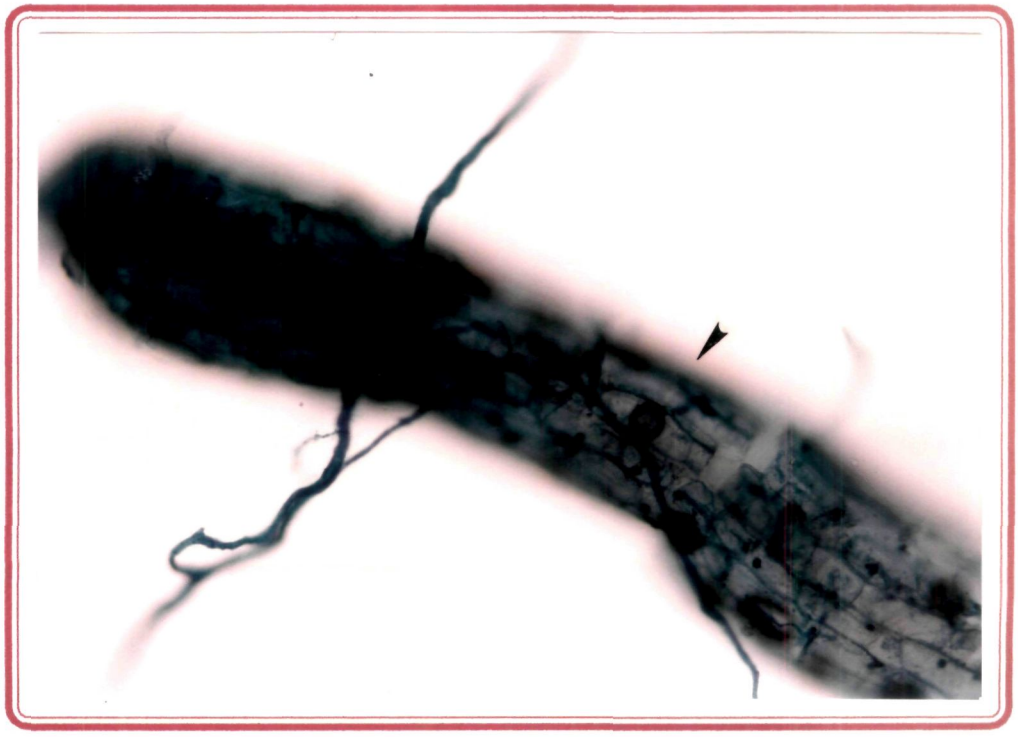
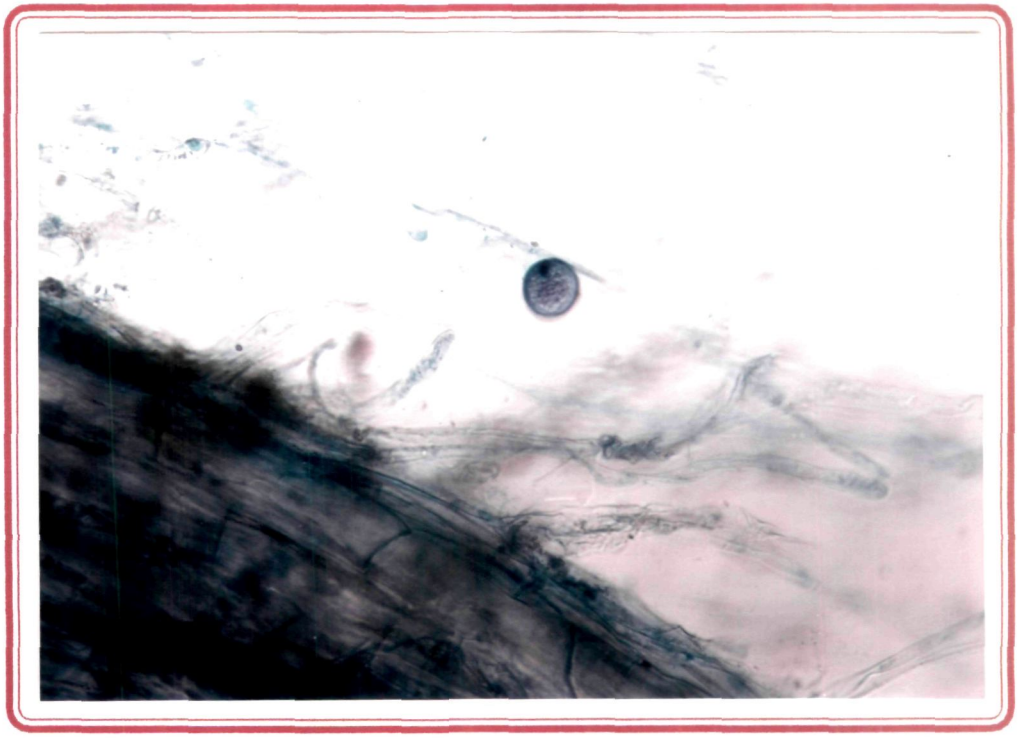


PLATE 25: Sporulation of *Gigaspora margarita* on the surface and outside the transformed tomato roots



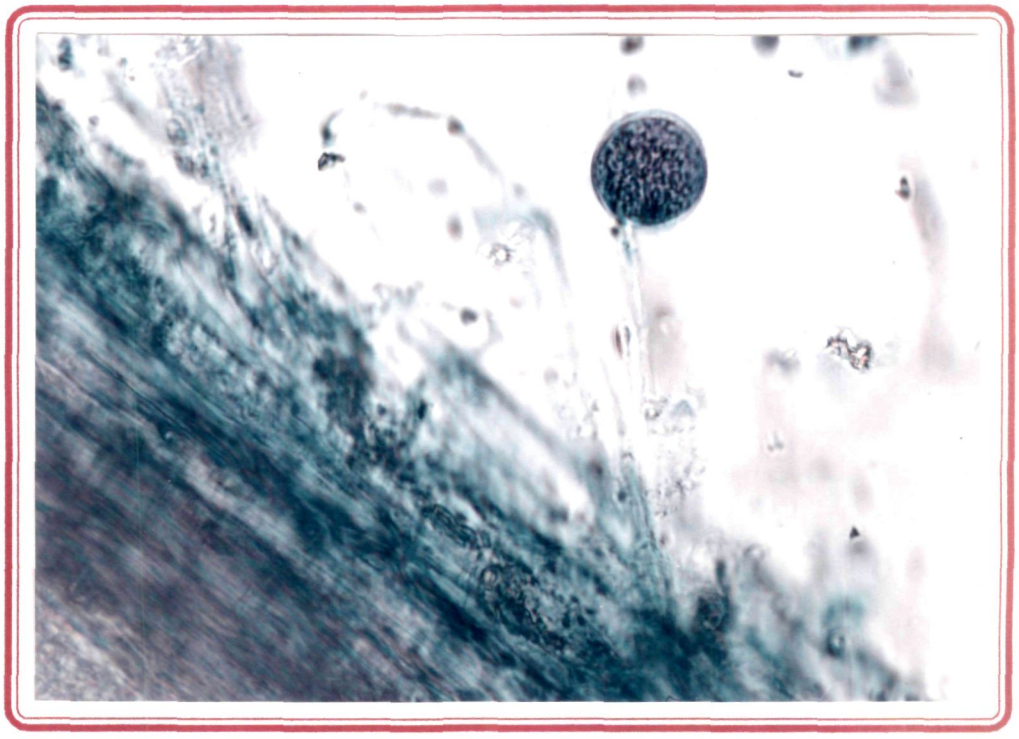


PLATE 26: Sporulation of *Glomus geosporum* in the transformed roots of tomato

DISCUSSION

V DISCUSSION

It is well documented that VA mycorrhizal fungi increase the growth and yield of most crop plants. The improved growth is attributed to increased in nutrient uptake, production of growth promoting substances, tolerance to drought, salinity and transplant shock, resistance to plant pathogens and synergetic interactions with beneficial soil microorganisms like nitrogen fixers, phosphate solubilizers etc. However, harnessing these beneficial effects for commercial utilization has proven difficult, since VA mycorrhizal fungi are obligate biotrophs and cannot be cultured on laboratory medium.

Several attempts have been made to culture these fungi on artificial medium with little or no success. Mosse in 1960's established that surface sterilized spores of VA mycorrhizal fungi can be used to infect plants under axenic conditions. In recent years, culture of isolated roots has been given a new impetus with the use of roots genetically transformed by the Ri – plasmid of *Agrobacterium rhizogenes* (Tepfer, 1984). Rapid growth of axenic hairy root led Mugnier and Mosse (1987) to inoculate such roots with VA mycorrhizal fungi. This was followed by a few more studies which demonstrated that root organ cultures have potential for growing VA mycorrhizal fungi *in vitro*.

All attempts have been made in recent years with *in vitro* cultivation or isolated roots especially the cultures of genetically transformed roots following

infection of plant tissue by *Agrobacterium rhizogenes*. Protocols have been developed for culturing of excised roots of different plant species. However, it has been pointed out there is a need for improving the protocol for the production of hairy roots (Becard and Piche, 1992). Thus, the present investigation was taken to modify the culture media, select the host species and the plant organ used in order to come out with the best suited method for rapid reproduction of transformed roots. This inturn can be used for culturing VAM fungi.

AT the present study, establishment of Ri T-DNA transformed roots was achieved by using wild strains of *Agrobacterium rhizogenes*. Ten different strains were collected from different laboratories. Each laboratory specified a culture medium best suited for the growth of that particular strain. Hence, an attempt was made to select a single medium suited for the growth of all the strains of *A. rhizogenes*.

Selection of a singe culture medium for culturing different strains of *A. rhizogenes* :

Ten strains of *A. rhizogenes* were cultured on four different culture media viz., yeast extract mannitol broth, yeast extract broth, modified yeast extract broth and nutrient broth. Of the four media studied, modified yeast extract broth was found to be the best supporting the growth of all strains of *A. rhizogenes*. Among the four media tested, modified yeast extract broth was the only medium with pH 6.8 while the other three media had pH 7.0. This suggests that a pH of 6.8 is better

suited than pH 7.0 for the culturing of *A. rhizogenes*. Mugnier (1988) found yeast extract mannitol medium at pH 6.8 is best suited for culturing two strains of *A. rhizogenes* ATCC – 31718 and A_4. Of the ten strains of *A. rhizogenes* tested, three strains namely LBA – 9402, ATCC – 15834 and A_4, which grew best, were selected for further studies.

Screening of different host explants for callus induction by *Agrobacterium rhizogenes* :

Ten different host plants namely *Mentha piperita*, *Mentha citrata*, *Ocimum gratissimum*, *Adhatoda vasica*, *Coleus aromaticus*, *Vigna radiata*, *Vigna unguiculata*, *Capsicum annum*, *Daucus carota* and *Lycopersicon esculentum* were tested for the induction of hairy roots using two different strains of *A. rhizogenes* on five different tissue culture media. Of the ten plants studied tomato and cowpea proved to be the best hosts for the induction of hairy roots. Among the stem, leaf petiole and leaf explants of the two host plants, stem was found to be the best plant organ for inducing hairy roots.

Leaf material is commonly used for inducing hairy roots (Mugnier, 1988 and Proter, 1991). It was interesting to observe that in the present study better rooting occurred in stem explants of both cowpea and tomato and also leaf petioles of cowpea. Tappi in 1997 reported maximum hairy root formation from stem explants of cucumber which is in support of the present observation. Both tomato and cowpea are dicots and it has been observed by earlier workers that dicots are better

suited for the production of hairy roots compared to monocots (Hamill *et al.*, 1987 and Tepfer, 1989).

One desirable characteristic of these transformed roots in their ability of quickly form numerous lateral roots. Tepfer (1984) concluded that they are better adapted to growth in culture than normal roots and that they also survive longer periods without subculture. Possibly the new genome of transformed roots affects its capacity for axenic production. Another characteristic sometimes observed in transformed roots is the inversion of their geotropic mode of growth. Nevertheless, it appears that transformed roots have the same synthetic capacity on roots of the plant from which they were obtained.

Screening of different host explants for callus induction by *Agrobacterium rhizogenes* on different tissue culture media :

Having selected the best strains of *A. rhizogenes* and the host for hairy root induction, an attempt was also made to select the best tissue culture medium for culturing transformed roots. Media used were MS medium, White's medium, modified White's medium, Gamborg B_5 medium and Nitch medium. In MS basal medium callus formation followed by rooting was maximum suggested it to be the best medium for the initial establishment of hairy roots. This is in conformity with the observations made by Toyok (1993) and Hosokawa *et al.* (1997) in barley and gentian plants respectively. However, B_5 medium was reported to be the best for hairy roots establishment of cowpea and some medicinal plants by Hamill *et al.*

(1987) and Zhou *et al.* (1997). Similarly Becard and Fortin (1988) observed modified White's medium to be the best for the production of transformed roots of carrot. These earlier studies compared to the present investigation suggests that the best medium for hairy root formation differs with the host.

Effect of different levels of sucrose in MS basal medium on growth of hairy roots :

Modification of the MS basal medium was attempted so that it can promote better growth of hairy roots. Sucrose is very critical for axenic culturing of tissues. Reduced sucrose level in the medium, having and adverse effect on hairy root formation has been reported by earlier workers. The present study brought out that sucrose in the concentration of 2.5 per cent in MS basal medium is ideal for hairy root formation. This was true not only for tomato but also for cowpea. Studying the different levels of sucrose in Gamborg B_5 medium, Christen *et al.*, 1992 reported that three per cent sucrose is best suited for the hairy root and alkloid production in *Hyoscyamus albus*.

Establishment of hairy roots by *A. rhizogenes* :

The studies done so far were for root induction. Having standardized the protocol for root induction, the next step attempted was to select a medium for best establishment of hairy roots. It has been observed that nutritionally richer medium is needed for hairy root induction in the initial stages, which has to transferred to a

less nutritious medium (after completely removing bacteria) for better establishment of roots at later stages so that it can support the VAM fungal colonization.

Out of the four different media namely, MS medium, 0.5 x MS medium, modified White's medium and minimal medium, minimum medium was found to the best suited for the establishment of hairy roots. Because of this reason White's medium with lesser nutrients is recommended for hairy roots establishment compared to MS medium which is considerably richer (Becard and Piche, 1992).

Compared to MWM (20%) and MS medium (30%), the level of sucrose in the minimal medium (MM) is less (10%). Higher levels of sucrose in the medium having a deleterious effect on VAM colonization has been reported by Mosse (1980). She also pointed out that a medium with less sucrose concentration will be the best suited for growing transformed roots and co-culturing it with VAM fungi. In the present study establishment of hairy roots was better in roots induced by *A. rhizogenes* strain LBA - 9402 followed A_4 and ATCC – 15834.

Establishment of tomato hairy roots by *A. rhizogenes* on different tissue culture media at 25 and 30^{0}C :

The effect of temperature (25 and 30^{0}C) on the establishment and growth of hairy roots was studied. Most of the earlier work has been done by incubating the plates for hairy roots development at 24 to 25^{0}C. The present study clearly brought out that there is no much difference between the growth of hairy roots in incubated

at 25 or 30^{0}C. This suggests that after initial root induction at 25^{0}C for 10 days, the plates can be incubated at higher temperature of 30^{0}C. It was observed that incubation at higher temperature after root induction resulted in less accumulation of water droplets in the plates, thus reducing contamination compared to plates incubated althrough at 25^{0}C. This is of special significance in places where the room temperature is around 30^{0}C, there by the plates can be incubated without an incubator. Root induction can also take at this room temperature of 30^{0}C but at a lower percentage as observed in a preliminary study.

Effect of growth regulators on the growth of tomato hairy roots induced by *A. rhizogenes* (Strains LBA – 9402) :

Some of the earlier workers suggested that addition of growth regulating substances to the culture medium can enhance hairy root formation (Robbins and Hervey, 1978). It was also expressed that hairy root production depended on the auxins present in the medium. Butcher and Street (1964) pointed out that when sugar concentration in the medium is reduced, it in turn will bring deficiency in the synthesis of growth regulators and thus emphasised the need for addition of growth regulators in the medium. The present investigation clearly brought out that addition of growth regulators (NAA, IBA and IAA) at any of the concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) studied did not support growth of hairy roots. On the contrary it brought in stunting and reduction of root growth.

Confirmation of Ri T-DNA transformation by opine assay :

Hairy root induced by *A. rhizogenes* synthesises opine (Tepfer and Tempe, 1991). The constitute cells have integrated copies of T-DNA which occurs in a longer plasmid of *A. rhizogenes*, the Ri – plasmid (Chilton, 1982). The different regions of T-DNA are known to encode for opines and auxins (Tepfer, 1989). Successful transformation of Ri – plasmid from the *A. rhizogenes* to the hairy roots can be confirmed by the detection of opines in root tissue (Tepfer and Tempe, 1981). The two major opines produced are agropine and mannopine (Costantin *et al.*, 1981). It has been reported agropine Ri – strains produced more abundant roots than the mannopine types (Petit *et al.*, 1983). In the present study successful transformation of Ri – plasmid was confirmed by detecting opines in the root tissue by high voltage paper electrophoresis (Petit *et al.*, 1983). The result indicated that the hairy roots induced by *A. rhizogenes* strains LBA – 9402, ATCC – 15834 and A_4 produced agropine.

Microscopic observation of hairy roots :

Very few microscopic observations have been made on the hairy root produced by *A. rhizogenes*. Such observation made in the present study revealed that the transformed roots grow much faster with more branches and hairy appearance compared to non-transformed roots. This upholds the earlier observations made by Dobigny *et al.* (1995) in potato transformed roots.

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Dual culture of hairy roots and VAM fungi :

VAM fungal inoculum :

Having the standardised the method for the establishment and growth of hairy roots attempts were made to inoculate VAM fungal spores on the hairy roots in order to obtain dual culture of root and fungus. For this purpose it is essential to have VAM spores with least contamination.

The fungal spores to be sterilized must be purified as much as possible since most of the contaminations come from either old spores or soil debris. So, VAM spores after extraction from soil by wet sieving and decantation (Gerdemann and Nicolson, 1963) should be subjected to density gradient centrifugation. By this density gradient centrifugation most of the debris and other dead spores are removed. The centrifugation method of Furlon *et al.* (1980) has been effectively used by most of the workers. The sucrose gradient was used for the method. After extraction if the spores are not washed thoroughly then the contamination of bacteria and fungi would be more. The other problem is that the gradients after being transferred to the centrifuge tubes, should be centrifuged quickly otherwise mixing of the gradients will take place. Hence, the possibility of replacing sucrose with glycerol was studied. The retrieval of spores using 40:80 and 30:60 gradients glycerol was more compared to sucrose gradients. The number of spores retrieved using 40:80 and 30:60 glycerol gradients was more or less the same not differing significantly from each other. Thus the present study brought out that glycerol can

be used instead of sucrose for retrieving VA mycorrhizal spores by density gradient centrifugation. Glycerol being a good preservative, the spores retrieved can perhaps be stored for a longer period.

VAM spore sterilization :

The success of axenic culturing of transformed root with VAM fungi is based on purity of the VAM spores used. Most of the contaminants are from the surface of the spores used for the study. Surface sterilized resting spores of VAM fungi infecting plants under axenic condition was first established by Mosse (1962). Since then many procedures have been used for surface sterilization (Mertz *et al.*, 1979; Tommerup and Kidby, 1980 ; Macdonald, 1981 and Strullu and Romand, 1986. Most scientists prefer Chloramine-T (2%) solution with surfactant and antibiotics such as streptomycin and gentamycin (Mosse, 1962). The success in utilizing this disinfectant solutions is higher depending on how they are used and on which material.

In the present study it was found sterilization of spores with chloramine-T (2%) for 10 to 15 minutes followed by antibiotics mixture of streptomycin (200 ml/l) and gentamycin (100 ml/l) for 2 to 3 minutes, followed by cefotoxime (250 mg/l) for 5 minutes resulted in complete sterilization of VAM spores. Use of cefotoxime for the surface sterilization of VAM spores was attempted for the first time through this work.

Effect of different concentration of cellulase on spores germination of VAM fungi :

The enzyme cellulase has been reported to enhance spore germination of VAM fungi (Clark, 1997). In the present study treatment of VAM spores at concentration of 16 mg/l and 8 mg/l significantly enhanced spores germination, while treating the spore at a higher concentration of 20 mg/l reduced spore germination.

Effect of different concentration of cell-free extracts of bacteria and fungi on VAM spore germination :

Cell-free extracts of beneficial soil bacteria such as *Rhizobium* and *Azotobacter* enhancing colonization of VAM fungi has been reported earlier (Azcon *et al.,* 1978). Similarly Machado and Bagyaraj (1995) using the MHB *Bacillus coagulans* found enhanced mycorrhizal colonization in cowpea. In the present study cell-free extracts of *Bacillus coagulans*, *Azospirillum brasilense, Azotobacter chroococcum* and *Rhizobium* sp. significantly enhanced spore germination of VAM fungi, thus upholding the observations made by earlier workers.

Cell-free extracts of fungi like *Aspergillus* sp. (LC_4), *Trichoderma* sp. (LC_5) and *Phenocheate chrysosporium* enhanced spore germination of VAM fungi. Fungi

like *Aspergillus* sp. enhancing spore colonization has been reported by Gopalakrishna (1980).

Establishement of dual culture :

Pregerminated VAM spores were used to inoculate transformed roots by placing them about 1 cm away from the tip of the emerging lateral roots. The germ tube of VAM fungi are negatively geotrophic and hence the plates were incubated vertically in order to bring better contact of the germ tube with the root system. The co-culturing was done at 25 and 30^{0}C. The observations were taken at periodical intervals revealed that there was no significant difference between the two incubation temperatures. This suggests that places where room temperature is around 25 to 30^{0}C, the co-culturing of VAM fungi can be done at root temperature.

Microscopic observations of hairy roots co-cultured with VAM fungi revealed extensive colonization of roots with abundant extramatricular hyphae. The percentage colonization of roots ranged from 16.45 to 33.50 per cent. Maximum percentage colonization being produced by *Gigaspora margarita*. The intraradical mycelial development within the root, though appeared faint without staining was very prominent after staining giving a beaded appearance to the root. Arbuscules appeared first after 15 to 18 days of incubation, vesicles appeared later (20 to 22 days) and spores appeared on the extramatricular hyphae after 35 to 40 days.

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In the present study improvements on the existing root organ culture method have been made to produce hairy roots and co-culture VAM fungi in them. The dual culture of VAM fungus in root organ culture will enable the following feasible in the near future :

1) maintenance of pure culture of VAM fungi in the laboratory.
2) structural as well as cytological analysis of hyphal and spore development of VAM fungi.
3) effect of environmental stresses like heavy metal toxicity, extremes of temperature and pH and competition with pathogens and other organisms.
4) isolation of DNA from hypha and spore of VAM fungi for molecular biological studies.
5) to understand the host specificity preference in VAM symbiosis.
6) in a long run, several R and D inputs may help in the commercial production of VAM fungi through bio-reactors.

SUMMARY

VI SUMMARY

An attempt was made to study the various factors that can improve the Ri T-DNA transformed hairy root production by *Agrobacterium rhizogenes*. Ten wild strains of *A. rhizogenes* obtained from different sources and maintained on different media were screened for the suitability to grow on a common culture medium. Modified yeast extract broth was found to be the best culture medium to grow most of the strains screened.

Ten host plants were selected and screened for the hairy root induction by *A. rhizogenes*. Different parts of the plant organs like leaf, leaf petioles and stem explants were tried for the development of hairy roots. Leaf petioles of cowpea and stem explants of tomato were found to be best suited for root induction. Out of the ten strains of *A. rhizogenes* tested three strains namely, LBA – 9402, ATCC – 15834 and A_4 resulted in maximum callus induction and root initiation. Further studies were carried out with hairy roots developing from tomato explants.

The suitable level of sucrose for the hairy roots in different tissue culture media was studied. Basal MS medium with 2.5% sucrose gave the best results. Increase in sucrose level led to the reduction in growth of hairy roots. For co-culturing hairy roots with VAM fungi, it is important to have a medium which will support the growth of both the biological systems, as rich media are not

favourable to the growth of VAM fungi. Hence, hairy roots initiated on MS basal medium were later transferred to minimal medium (MM) so that it can support the growth and establishment of both the root as well as the VAM fungus.

Establishment of transformed root on minimal medium was studied at 25 and 30^0C. There was not significant difference between the two incubation temperatures. Transformed hairy roots were thicker, grew faster with large number of root hairs compared non-transformed control roots. The transformed roots covered the entire petriplate in 25 to 30 days.

Addition of growth regulators namely, IAA, IBA and NAA at concentrations 0.5, 1.0, 1.5 and 2.0 mg/l to the culture medium adversely affected root growth at all concentrations.

The transfer of Ri T-DNA from *Agrobacterium rhizogenes* to hairy roots was confirmed by opine assay carried out through high voltage paper electrophoresis. The result confirmed the presence of agropine in transformed roots induced by all the three strains of *A. rhizogenes* (LBA – 9402, ATCC – 15834 and A_4).

Dual culture of transformed roots and VAM fungi was carried out by using VAM fungi *Glomus fasciculatum, Glomus mosseae, Gigaspora margarita* and *Glomus geosporum*. Success of such dual culture lies in the purity of VAM spores used for the inoculation. After the isolation of VAM spores by wet sieving and

decantation, the spores are usually purified by sucrose gradient centrifugation, which encourages contamination when sucrose is not washed properly. An alternate method namely, glycerol gradient centrifugation was found to be the best for retrieval of maximum VAM spores and resulted in least contamination.

A predominant VAM fungus from mine spoil was isolated, purified, brought into pot culture and identified up to species level as *Glomus geosporum*. This fungus was also included in the co-culturing studies.

Since, pregerminated spores are usually used for inoculating hairy roots, cell-free extracts of certain bacteria and fungi and also different concentrations of the enzyme cellulase were tested for their effect on spore germination of the selected VAM fungi. Cell-free extracts of *Azospirillum brasilense, Trichoderma* sp.(LC_5) and cellulase enzyme at 16 mg/l concentration resulted in maximum spore germination.

Microscopic observations of dual culture were carried out at periodical intervals. The intraradical mycelial development was clearly seen after 12 to 15 days. Arbuscules appeared after 15 to 20 days and vesicles after 20 to 22 days. Sporulation occurred in the extramatricular mycelium after 30 to 50 days of establishment. Dual culture was best in roots induced by *A. rhizogenes* strain LBA – 9402 colonized by *Gigaspora margarita*.

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* : Original not seen.