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INTEGRATION OF BIOINOCULANT TECHNOLOGY WITH MICROPROPAGATION OF BLACK PEPPER (*Piper nigrum* L.) AND STANDARDIZATION OF PRODUCTION OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF) IN TRANSFORMED ROOTS

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

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Faculty of Agriculture
Kerala Agricultural University, Thrissur

2004

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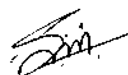
DECLARATION

I hereby declare that this thesis entitled "**Integration of bioinoculant technology with micropropagation of black pepper (*Piper nigrum* L.) and standardization of production of arbuscular mycorrhizal fungi (AMF) in transformed roots**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.



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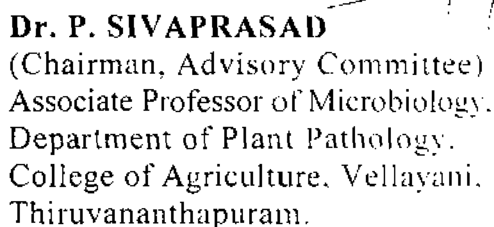

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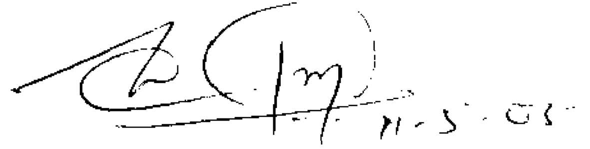

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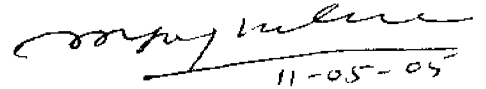

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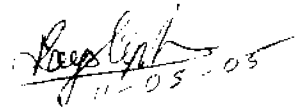

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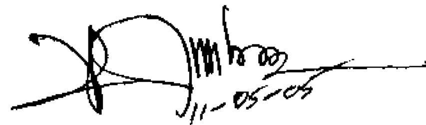

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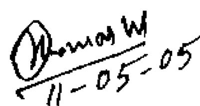

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INTRODUCTION

1. INTRODUCTION

One of the important applications of modern biotechnology is its use in tissue culture. *In vitro* micropropagation techniques are increasingly being applied for large-scale production of quality planting materials. Of the four important stages in micropropagation, regeneration *in vitro*, proliferation of the regenerates, rooting *in vitro* and transplantation to out door, the last stage is very crucial and important with respect to the establishment of *in vitro* derived plantlets.

Tissue culture plantlets have very divergent leaf anatomy and physiology and hence require an acclimatization period during the transition from culture to green house, from a total unnatural system to the very natural environment. The plantlets may not be able to withstand such sudden shock of the environmental changes. One of the major impediments to the success of micropropagation is the very high mortality rate of *in vitro* plantlets either during acclimatization phase or during transfer to the field conditions. Desiccation, wilting and pathogenic infection are main causes of low survival. It is estimated that only about 25 per cent of *in vitro* regenerated plantlets are successfully transplanted *ex vitro* and still fewer establish into the field (Sivaprasad and Sulochana, 2004). Such a disappointing state of affairs has been attributed to certain aberrant physiological features characteristic of *in vitro* derived plantlets.

The poor development of cuticular wax of plants developed under high humidity condition and the abnormal orientation of micro fibrils and defunct stomatal system leads to excessive dehydration and wilting of plantlets (Lesham, 1983; Capellades *et al*, 1990). The micropropagated plantlets, during early stages, often fail to compensate this water loss through effective water absorption and transportation as the root hair is not properly developed (Reuther, 1986) and the vascular connection between root and shoot is defective (Sivaprasad and Sulochana, 2004). This ultimately ends up with heavy mortality due to dehydration and wilting. Further, plantlets developed under controlled aseptic conditions are highly vulnerable to microbial infection, as their defense mechanism has not been triggered earlier. Any technology that helps to improve the water uptake, transport and

maintain high water potential of the plant along with protection against pathogenic infection and growth promotion will be of immense application in micropropagation.

Black pepper (*Piper nigrum* L.) popularly known as 'king of spices' is the most important spice crop of India. India is a leading producer and exporter of black pepper. Kerala alone contributes about 97.2 per cent of the total area in India (Parayil and Aipe, 2002). In spite of the steady increase in area under black pepper cultivation in Kerala, there is no corresponding increase in production. The reason for poor productivity of pepper can be attributed to various factors including diseases, pests and nutrition. Of these, foot rot disease incited by *Phytophthora capsici* is identified as the major production constraint in India. The fungus irrespective of the age of the plant, infects roots, stem as well as leaves of black pepper and causes a loss of 30 to 100 per cent vines (Dutta, 1984).

Micropropagation is being extensively used for the production of disease free, quality plantlets of black pepper for distribution to the farmers. The high mortality and slow growth of the plantlets during acclimatization (hardening) phase is a major constraint. This mortality is attributed mainly to the physiological defects of the plantlets as well as infection by foot rot pathogen, *P. capsici*. The micropropagation technique usually does not take into consideration the existence of mutualistic symbiosis of mycorrhiza and other associative Plant Growth Promoting Rhizobacteria (PGPRs). An early inoculation of micropropagated plants with appropriate symbiotic organism promises to improve plant survival and performance (Lovato *et al*., 1996). The soil microorganisms associated with plant species influence the plant growth and development in many ways. Some organisms invade the plant and establish mutualistic relationship and often the plants are benefited in terms of better nutrition and protection against diseases. Mycorrhiza, endophytic *Pseudomonas* and *Azospirillum* are known for such interactions. Mycorrhizal hyphae invade the root; ramify inside and the mycelia spread towards the soil. The mycelium help in the uptake and transport of nutrients and water from the soil. It also brings about physiological and biochemical change in the host that confers improved growth and better tolerance to pathogenic infection (Bolan *et al*., 1987; Davies *et al*., 1993; Sivaprasad *et al*., 2000; Sivaprasad and Sulochana, 2004). It is

possible to correct many of the deficiencies of tissue culture plantlets with the desirable traits of mycorrhizal association. Fluorescent pseudomonads, in addition to direct inhibition of pathogens through the production of highly potent inhibitory metabolites, enters the plant system and produce a spectrum of metabolites which induces systemic resistance in plants against pathogenic infection (Ramamoorthy *et al.*, 2000). They are also known for the production of growth hormones and improve the root development and growth of the plant (Kloepper *et al.*, 1980b; Arshad and Frankenberger, 1998). *Azospirillum* is an associative diazotroph that helps in the nitrogen nutrition of the plant and also improve the growth and development of the plant through hormone production (Okon *et al.*, 1976; Varma, 1995). Black pepper plants were also known to associate with microorganisms such as AMF and fluorescent pseudomonads, which resulted in improved growth and disease tolerance (Sivaprasad *et al.*, 1995b; Sarma *et al.*, 1996; Sivaprasad *et al.*, 2003). In the present investigation, attempts were made to integrate microbial inoculants such as AMF, *Pseudomonas* and *Azospirillum* to improve the survival, growth characteristics and foot rot disease tolerance of black pepper plantlets.

In order to exploit these microbial inoculants in the micropropagation programme, availability of quality inoculum is an important factor. Although *Pseudomonas* and *Azospirillum* can be multiplied and produced aseptically, there is no proper technology for inoculum production under aseptic conditions for mycorrhiza, as it is an obligate symbiont. *Agrobacterium rhizogenes* mediated transformed root culture for mycorrhizal inoculum production is being attempted by many workers (Chabot *et al.*, 1992; Khaliq and Bagyaraj, 2000; Fortin *et al.*, 2002). The successful co-culturing of transformed roots with mycorrhiza enables the production of mycorrhizal inoculum aseptically.

The present investigation was undertaken with following objectives:

(1) Evaluation of the effect of inoculation with AMF, *Azospirillum* and fluorescent pseudomonads in different combinations on establishment, growth and foot rot tolerance of micro propagated black pepper plantlets.

(2) Standardization of transformed root culture technique using *Agrobacterium rhizogenes* for AMF inoculum production in selected host plant.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Tissue culture is considered as an important tool for cloning and propagation of crop plants. This technique not only increases the scale and speed of production, but also yields healthier plantlets. It has got several advantages over conventional methods of plant propagation with application in horticulture, agronomy and forestry. Today about 50 per cent of flori-horticultural plants are produced through micropropagation techniques (Grotkas *et al.*, 2000).

The most important problem associated with micropropagation is the establishment of *in vitro* derived plantlets under outdoor conditions, which limits the widespread use of the technology. Tissue culture plantlets show certain aberrant physiological features which lead to very high mortality due to desiccation and microbial infection either during hardening or during transfer to field conditions (Capellades *et al.*, 1990).

2.1 PHYSIOLOGICAL FEATURES OF TISSUE CULTURE PLANTS

The tissue culture plantlets when planted out undergo desiccation and drying due to poor development of cuticle and epicuticular wax on the newly emerging leaves, as a result of high humidity in the culture vessels (Lesham, 1983). The palisade cells of leaf surface are poorly developed and have pronounced mesophyll air spaces (Donelly and Vidaver, 1984). The plantlets are photomixotropic and have leaves with low chlorophyll content and low photosynthetic rates that impede growth due to poor organization of grana in the chloroplasts of the *in vitro* growing plantlets along with etiolated effect produced by ethylene in the glass vessels (Grout and Aston, 1977; Lee *et al.*, 1985). The micropropagated plantlets show an impaired stomatal mechanism (Capellades *et al.*, 1990) due to which transpiration rates are initially very high, which results in wilting, necrosis of leaves and may include senescence and death of leaves and plantlets (Preece and Sutter, 1991). A percentage of cultures show water soaked, almost translucent leaves and exhibits a decline in growth and multiplication

(Debergh *et al.*, 1981). Lack of proper root hair development in tissue culture plants in general directly affects the absorption of nutrients and water from the soil (Reuther, 1986). These major physiological defects of tissue culture plantlets affect uptake of water and nutrients from soil and transportation to the shoot system and the plantlets become more vulnerable to desiccation and wilting and also to pathogenic infection (Sivaprasad and Sulochana, 2004).

2.2 DESIRABLE TRAITS OF AMF

Arbuscular mycorrhizal fungi (AMF) are soil fungi which form symbiotic association with plants and colonize roots of most of the plant families (Smith and Read, 1997). Arbuscular mycorrhizal associations are formed by a group of zygomycetous fungi belonging to the order glomales (Morton and Benny, 1990). They impart a variety of benefits to their hosts, which include increased growth and yield due to enhanced nutrient acquisition (Diedrichs and Moawad, 1993), water relations (Davies *et al.*, 1993; Subramanian *et al.*, 1995), pH tolerance (Clark and Zeto, 1996) and disease and pest tolerance (Lopez *et al.*, 1997).

2.2.1 Improved Nutrient Uptake

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

2.2.2 Improved Water Relations

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

2.2.3 Drought Tolerance

AMF association enhances tolerance of plants to toxicity and drought (Atkinson and Davidson, 1972; Guttay, 1976; Ruiz-Lozano *et al.*, 1999; Davies *et al.*, 2002). Mycorrhiza induced drought tolerance can be related to factors influenced by AM colonization such as improved leaf water and turgor potentials (Al-Karaki, 1998; Sanchez-Blanco *et al.*, 2004), maintenance of stomatal functioning and transpiration (Estrada-Luna and Davies, 2003; Sanchez-Blanco *et al.*, 2004), greater hydraulic conductivities and root development (Sivaprasad and Sulochana, 2004). AMF treated plants fully recovered plant photosynthetic activity under drought stress (Borrowska, 2002; Sivaprasad and Sulochana, 2004). Mycorrhizal colonization was found to improve water use efficiency and plant yield in watermelon (Kaya *et al.*, 2003).

2.2.4 Well Developed Root System

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

micropropagated plants of pineapple. AMF colonized micropropagated sugarcane plants showed excessive root growth and root dry weight, which results in better survival of these plants (Gosal *et al.*, 2001).

2.2.5 Salt and Heavy Metal Tolerance

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

2.2.6 Improved Soil Structure

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

2.3 PHYSIOLOGICAL AND BIOCHEMICAL CHANGES DUE TO AMF COLONIZATION IN HOST PLANT

Sutter (1988) studied stomatal and cuticular water loss during acclimatization in apple, cherry and sweet gum plantlets and found that in acclimatized plantlets, stomatal conductance of persistent leaves decreased to about half of that in the *in vitro* leaves while cuticular conductance remained the same. He concluded that increased stomatal closure reduced the conductance. The capability of *in vitro* stomata to adapt to the new environmental conditions by modifying guard cells during acclimatization enlighten the role of stomata in death of micro propagated *Prunus cerasus* plants after their transfer to the external environment (Martin *et al.*, 1988). Mycorrhizal pepper plants show high net photosynthetic flux, tissue P concentration, stomatal conductance or leaf turgor during high environmental stress or recovery from stress, which indicated superior drought resistance of these plants (Davies *et al.*, 1993). The stomatal conductance was increased by *Glomus fasciculatum* in unimproved genotype of maize (Aguilera-Gomez *et al.*, 1998). The mycorrhizal plants were more water use efficient than non-mycorrhizal plants (Al-Karaki, 1998). According to Hernandez-Sebastia *et al.* (1999) root colonization by *Glomus intraradices* modifies the water status, control of water losses and osmotic relations of micro propagated strawberry plantlets under *in vitro* conditions of high humidity. Endomycorrhizal (*Glomus intrardices*) colonization in Chile ancho pepper alleviated low P effects by increasing net photosynthetic rate, stomatal conductance, P use efficiency and decreasing internal CO_2 concentration (Aguilera-Gomez *et al.*, 1999). Micro propagated banana plants when inoculated with AMF during acclimatization phase show increased nutrient level, photosynthesis and transpiration rate, water potential and stomatal conductance (Yano-Melo *et al.*, 1999). AMF colonized Chile ancho pepper plantlets showed lower abscisic acid and higher relative water content than non-AMF plantlets, during peak plant dehydration (Estrada-Luna and Davies, 2003). Under drought stress, mycorrhizal *Olea europaea* seedlings showed significantly higher photosynthetic and transpiration rates, stomatal conductance and foliar P concentration, than its similarly sized non-mycorrhizal

counterpart (Caravaca *et al.*, 2003). Higher photosynthetic rate and stomatal conductance as early as days five and seven after inoculation with AMF than non-mycorrhizal plantlets were noticed in Chile ancho pepper (Estrada-Luna and Davies, 2003). *Rosmarinus officinalis* plants when inoculated with *Glomus deserticola* improved photosynthetic activity and stomatal conductance under water stress when compared to non-mycorrhizal stressed plants (Sanchez-Blanco *et al.*, 2004). The photosynthetic activity of plants is improved due to AMF colonization (Sivaprasad and Rai, 1985; Yano-Melo *et al.*, 1999; Dell'Amico *et al.*, 2002; Caravaca *et al.*, 2003; Sanchez-Blanco *et al.*, 2004). The reduction in stomatal and mesophyll resistance to carbon dioxide uptake, increased chlorophyll content and better hydration of plants brought about by AMF colonization favors CO_2 fixation (Sivaprasad and Sulochana, 2004). Root colonization by AMF results in increased vegetative growth, total chlorophyll content and uptake of nutrients by host plant (Mathur and Vyas, 1999; Sanchez-Blanco *et al.*, 2004).

Phenolic compounds in plants were considered to be preformed inhibitors of pathogens, which play a significant role in disease resistance (Mahadevan, 1970). According to Bhatia *et al.* (1972) resistance to pathogens has been correlated with the phenol content of roots. The mycorrhizal inoculation has been reported to impart resistance to the host against a spectrum of diseases (Dehne and Schonbeck, 1979). Nemec and Meridith (1981) showed abundance of phenols especially orthodihydric (OD) phenols in AMF structures. Mycorrhiza treated plantlets showed increased resistance to soil borne pathogens due to higher production of phenolic compounds including phytoalexins within the plant system (Hussey and Rancodori, 1982). Inoculation of peanuts with *G. fasciculatum* conferred resistance against *Sclerotium rolfsii* attack, which was related to the higher phenolic content in the host tissue (Krishna and Bagyaraj, 1983). A continuous increase of total soluble phenols in arbuscular mycorrhizal roots of *Arachis hypogea* was also reported by Krishna and Bagyaraj (1984). Histochemical studies on mycorrhizal plants showed enhanced phenolic accumulation on groundnut roots at early stages of mycorrhiza formation (Krishna

and Bagyaraj, 1986). Sivaprasad and Rai (1987) reported higher activity of phytohormones like cytokinin and indole acetic acid in plants inoculated with AMF, which results in better growth and development. Increased phytohormone activity due to AMF was also reported by Allen *et al.* (1982), Barea and Azcon-Aguilar (1982), Danneberg *et al* (1992) and Goicoechea *et al.* (1995).

Mycorrhizal inoculation resulted in a significant enhancement of percentage of colonization, dry matter and phenolic content of sesame plants in sterilized soils and this could be due to the contribution of AM fungal structures (Selvaraj and Subramanian, 1990). Grandmaison *et al.* (1993) found no qualitative difference in the soluble and bound phenolics isolated from non-mycorrhizal *Allium cepa* roots with those from mycorrhizal roots but the mycorrhizal roots showed higher concentration of wall bound phenolic compounds. Binding of phenolic compounds to cell wall could be responsible for the resistance of AM roots to pathogenic fungi, as it results in increased resistance by the cell wall to the action of digestive enzymes. Increased total phenol content in maize plants due to *G. fasciculatum* inoculation has been reported (Chabra and Jalali, 1995). The total phenol and orthodihydroxy phenol content of tissue culture plantlets of jack was significantly increased due to AMF inoculation (Sivaprasad *et al.*, 1995a). High phenol content in black pepper plants was also reported by Sivaprasad *et al.* (2000). AMF colonization alleviated the physiological effects of soil phosphate deficiency and induced changes in root phenolics and then regulates the degree of mycorrhizal colonization (Ganz *et al.*, 2002).

Higher rates of amino acids and reducing sugars in the plant root exudates was correlated with enhanced AMF colonization resulting in subsequent disease suppression (Graham *et al.*, 1981). Synthesis of new proteins or lytic enzymes in pea tissues on inoculation with mycorrhizal fungi hydrolyzed the polymers of cell wall and was considered as a defense response to invasion by parasitic organisms (Mauch *et al.*, 1988). According to Rao and Rao (1998), total soluble sugar concentration was higher in mycorrhizal plants of black gram and green gram than non-mycorrhizal plants. Boucher *et al.* (1999) showed that soluble protein concentration was remarkably increased with *Glomus etunicatum* colonization in

maize plants. In clover seedlings under salt stress conditions, the intensity of salt stress showed a positive correlation with sugar accumulation for the plants inoculated with AMF (Khaled *et al.*, 2003). Nodulated clover seedlings inoculated with AMF showed antagonistic interaction while in seedlings inoculated with AMF alone, phosphate nutrition was improved which help in salt tolerance. Both types of symbiosis helped to increase leaf protein content (Khaled *et al.*, 2003).

2.4 INTEGRATION OF AMF TECHNOLOGY WITH TISSUE CULTURE

Studies have proved that successful hardening and *ex vitro* establishment of micro propagated plantlets could be achieved by inoculation with AMF at the time of planting out (Lovato *et al.*, 1996). One of the earliest reports by Granger *et al.* (1983) on the effect of AMF (*Glomus epigaeum*) on apple clones suggested that the growth and leaf mineral content of two apple clones propagated *in vitro* were increased substantially with AMF inoculation. The growth of *in vitro* cultured strawberry plants were also enhanced due to the association of AMF (Kiernan *et al.*, 1984). The rooting of plantlets regenerated from callus was significantly enhanced by inoculation with *Glomus mosseae* (Fogher *et al.*, 1986). The transplant success and growth of *Robus idaeus* plantlets due to AMF inoculation was also reported by Pierik (1987). Mycorrhizal establishment significantly improved establishment, growth and mineral nutrition of oil palm plantlets (Blal and Gianinazzi-Pearson, 1988). The effect of AMF inoculation in micropropagated *Populus deltoides* was evaluated and it was found that rooted and hardened plants were best suited for AMF inoculation than other stages and also a positive response and colonization was obtained in terms of active root growth (Adholeya and Cheema, 1990). Micropropagated jack plantlets showed increased growth, survival percentage of 80-100 per cent and increased uptake of all the elements except iron as a result of AMF association (Ramesh, 1990). Micropropagated banana plantlets inoculated with *Glomus mosseae* or *Glomus monosporum* enhanced plant growth, P and N uptake and biomass production (Rizzardi, 1990). According to Sreelatha (1992) AMF association enhanced survival, growth characteristics and uptake of nutrients in micropropagated

anthurium. Inoculation with AMF seems to be the key factor for subsequent growth and development of micropropagated plantlets of avocado (Vidal *et al.*, 1992). Attempt to establish rose plantlets using AMF inoculation was met with considerable success (Wilson, 1993). Inoculation of micropropagated banana plantlets with *Glomus mosseae* and *G. geosporum* resulted in greater fresh and dry weights of shoots and higher P and K content (Declerck *et al.*, 1994). *In vitro* propagated cherry plants showed better growth and biomass production due to inoculation with AMF (Lovato *et al.*, 1994). Sivaprasad *et al.* (1995a) observed enhanced survival rate, growth and phenol activity in AMF inoculated jackfruit plantlets. Inoculation with *Glomus deserticola* on micropropagated plantlets of cassava in the post vitro, weaning stage enhanced per cent survival, tolerance to transplanting stress and shoot, root and tuber development (Azcon-Aguilar *et al.*, 1997). *Glomus constrictum* significantly improved the biomass production, nutrient uptake and acclimatization of *in vitro* plantlets of *Zizyphus mauritiana* into the field (Mathur and Vyas, 1999). Banana plantlets inoculated with mycorrhizae during the weaning stage of micropropagation produced a more efficient root system for the uptake of phosphates and other nutrients (Severn-Ellis, 1999). Application of *Glomus etunicatum* on micropropagated *Baptisia tinctoria* promoted plant survival which is of particular interest in the propagation of pharmaceutically important clones (BK 36, BK 37) as the higher survival rates would make commercial plant production economically feasible (Grotkas *et al.*, 2000). Micropropagated *Allium sativum* plantlets inoculated with *Glomus mosseae* exhibited better growth than control plantlets under *ex vitro* conditions (Lubraco *et al.*, 2000). *Glomus fasciculatum*, *G. etunicatum* and a native *Glomus* sp. inoculation on tissue culture plantlets of banana showed that AM colonization significantly increased the establishment rate, growth, vigour and biomass production during acclimatization and after transplanting to pots along with remarkable increase in P and Zn content due to *G. fasciculatum* colonization (Sivaprasad *et al.*, 1999b). A significant increase in growth, vigour and biomass production during acclimatization and after transplanting to pots was observed in tissue culture plantlets of alocasia when inoculated with *Glomus* sp.

and *G. fasciculatum* (Sivaprasad *et al.*, 1999a). Yano-Melo *et al.* (1999) also found that micropropagated banana plantlets inoculated with AMF had greater height, leaf area and fresh weight of shoots and roots, as well as higher rates of photosynthesis and transpiration than control plantlets. AMF inoculation increased the number of runners and daughter plants in micropropagated strawberry plants than control plants along with significant increase in N and P content in daughter plants (Alarcon *et al.*, 2000). Inoculation with *Glomus manihotis* inoculation significantly increased plant growth, root growth and nutrition of micropropagated cassava plants and also increased the field adaptability of plants, especially in marginal soils (Calderon *et al.*, 2001). According to Gosal *et al.* (2001) AMF infected sugarcane plants had better survival and excessive root growth upon transfer to soil. Taylor and Harrier (2001) reported that AMF improves mineral nutrition of micropropagated strawberry plants. Under drought stress conditions, AMF treated micropropagated plantlets of strawberry fully recovered their photosynthetic activity when watering was restored when compared to non inoculated plants (Borrowska, 2002). Plantlets of coffee inoculated with *Glomus clarum* increased plant height, leaf pair number, root number and also exhibited significant difference in enzymatic activities than un inoculated plants (Fernandez *et al.*, 2002). Cultivars of micropropagated banana showed high relative mycorrhizal dependency (RMD) values and also high N, P and K contents (Jaizme-Vega *et al.* 2002). Micropropagated taro plants after inoculation with AMF showed increased survival rate, contents of N, P, K, Cu and Zn in tissue of roots and leaves (LiMin *et al.*, 2002). Zeleznik *et al.* (2002) observed that transfer of micropropagated plantlets of yellow gentian into sterile substrate with mycorrhizal inoculum produced higher number of shoots that survived compared to transfer to sterile substrate with sterile mycorrhiza or into sterile substrate without mycorrhiza. Growth and development were faster in mycorrhiza treated *Echinacea pallida* plantlets than in non treated ones and the presence of well formed arbuscules and vesicles in infested roots in addition to 90 per cent success in the survival rate of vigorous plants indicated that mycorrhization is a valuable tool to overcome

Echinacea acclimatization shock (Lata *et al.*, 2003). *Gigaspora margarita* promoted growth, mineral nutrition and mycorrhizal colonization of micropropagated banana plants in different stages of rooting (Lins *et al.*, 2003). Micropropagated seedlings of banana when inoculated with *Gigaspora margarita* in the acclimatization phase resulted in high mycorrhizal colonization and increased growth (Trindade *et al.*, 2003). AMF inoculated micropropagated Chile ancho pepper plantlets had greater transpiration rates, photosynthetic rates, leaf chlorophyll content, leaf elemental N, P and K content, leaf dry biomass, leaf area and fruit production when compared with non AMF plantlets (Estrada-Luna and Davies, 2003).

2.5 CROP RESPONSE TO *AZOSPIRILLUM*

Plant growth promoting rhizobacteria like *Azospirillum* were known to contribute significantly towards plant establishment, growth and disease tolerance. The beneficial effect of *Azospirillum* on plant growth is by way of associative N fixation, capability to produce plant growth hormones and their effect on root morphology (Okon, 1985). *Azospirillum* inoculated plants showed increase in number of lateral roots with dense root hairs, root volume and root dry weight (Dewan and Rao, 1979). Inoculation with *Azospirillum* could induce rooting in black pepper cuttings (Govindan and Chandy, 1985). Inoculation with *Azospirillum* increased root development, growth and biomass production in crop plants in the nursery and field (Hadas and Okon, 1987). *Azospirillum* inoculation increased root length, root dry weight and total leaf area of tomato plants (Hadas and Okon, 1987), root growth, root volume, plant height, girth and number of leaves of bhindi (Parvatham *et al.*, 1989). Govindaswamy *et al.* (1992) reported an increase in total grain yield and straw yield of rice due to *Azospirillum* inoculation. Inoculation of *Azospirillum* in cabbage gave significantly higher yield than untreated control (Jeevajothi *et al.*, 1993). Significant increase in plant height, stem girth, root length and root weight was recorded due to inoculation with *Azospirillum brasilense* in coffee seedlings (Swarupa, 1996). Seed inoculation of *Azospirillum brasilense* in wheat plants increased the leaf area,

chlorophyll concentration, nitrate reductase activity, total biomass production and grain yield compared to untreated control (Panwar and Singh, 2000). Ability of *Azospirillum* to fix N has been confirmed by many workers (Lakshmikumari *et al.*, 1976; Okon *et al.*, 1976). The nitrogen fixation of the bacteria was found to be in the range of 20-24 mg N g^{-1} of carbon source (Okon *et al.*, 1976). *Azospirillum* strains produce phytohormones such as IAA, gibberellin and cytokinin like substances, which play an essential role in *Azospirillum* plant interaction (Tien *et al.*, 1979). The phytohormones induced root hair multiplication, shortening and thickening of roots in monoxenic cultures (Umali-Garcia, 1978). Varma (1995) observed that *Azospirillum* isolates from pepper produced maximum quantity of IAA equivalent to 69 μg ml^{-1}. The combined inoculation of selected native AMF cultures and *Azospirillum* isolates in chilly plants improved growth and suppressed damping off disease. Even though *Azospirillum* had no direct effect on disease suppression, dual inoculation of AMF and *Azospirillum* could suppress the disease (Kavitha, 2001). Incorporation of *Azospirillum* bioformulation increased root and shoot growth, dry weight and total N of both vegetativeiy propagated plants and seedlings of tea and also produced growth hormones like IAA and GA_3 in soil (Baliah *et al.*, 2003).

2.6 INTERACTION OF *AZOSPIRILLUM* WITH AMF

Pacovsky *et al.* (1985) reported synergistic interaction between AMF and *Azospirillum* in many crop plants, which leads to further increase in plant growth characteristics. Soil inoculation with *Glomus mosseae* and *G. fasciculatum* along with *Azospirillum brasilense* produced significantly higher dry matter production and grain yield in barley (Rao *et al.*, 1985). Inoculation of sweet potato cuttings with AMF and *Azospirillum* significantly increased the growth, plant N and P content, tuber weight and starch content (Kandasamy *et al.*, 1988). Dual inoculation of maize with *A. brasiliense* and *G. fasciculatum* significantly increased plant growth, yield and dry matter (Sreeramulu *et al.*, 1988). Combined inoculation of *Azotobacter*, *Azospirillum* and AMF in black pepper resulted in increased plant height and shoot and root weight compared to control (Bopaiah

and Khader, 1989). Inoculation of *Gigaspora margarita* and *A. brasilense* in coffee seedlings resulted in increased shoot length, root length, total dry weight and uptake of N, P and micronutrients such as Fe, Cu, Zn and Mn (Kumari and Balasubramanian, 1993). Murumkar and Patil (1996) found that combined inoculation of AMF and *Azospirillum* gave the highest yield of 19.1 t ha^{-1} in bell pepper. Dual inoculation of AMF and *A. brasiliense* increased the growth, yield and oil content of palmarosa significantly over uninoculated control (Neelima and Janardhanan, 1996). Kennedy and Chellapillai (1998) found that combined inoculation of AMF and *Azospirillum* showed increased height, total dry weight, AMF colonization and total N and P uptake in Shola tree species. Growth and nutrient content of black pepper cuttings were significantly higher when inoculated with *Glomus* sp., *Azospirillum* and phosphobacteria (Kandiannan *et al.*, 2000). The importance of biofertilizers such as AMF and *Azospirillum* in improving the supply and availability of nutrients in varying cropping systems of India has been explained by Pande and Gupta (2000).

2.7 *AZOSPIRILLUM* IN TISSUE CULTURE

Not much attempt has been made so far to make use of *Azospirillum* in micropropagation. A preliminary study conducted in banana and alocasia plantlets with AMF and *Azospirillum* inoculation showed better survival, growth and vigour of plantlets during hardening (Sivaprasad *et al.*, 1998).

2.8 FLUORESCENT PSEUDOMONADS FOR CROP GROWTH AND HEALTH

Plant growth promoting rhizobacteria such as fluorescent pseudomonads are known to contribute significantly towards plant establishment, growth and disease tolerance of crop plants (Suslow and Schroth, 1982). The use of PGPR as biofertilizers for growth promotion of crop plants is gaining much attention. Fluorescent pseudomonads may promote plant growth by producing phytohormones like auxins and gibberellins (Loper and Schroth, 1986). Barea *et al.* (1976) obtained 17 isolates of *Pseudomonas fluorescence*, which produced IAA, gibberellins and cytokinins. Enhancement of plant growth has been

attributed to yellow green, fluorescent siderophores produced by fluorescent pseudomonads (Kloepper *et al.*, 1980a). The growth promotion of winter wheat was achieved by treating with several strains of *Pseudomonas* spp. in green house and field conditions (Defreitas and Germida, 1992). Rangheswaran and Prasad (2000) reported increased growth in chickpea seeds treated with *P. fluorescence*. Fluorescent pseudomonads are known to produce considerable quantity of growth hormones that helps in the growth and root development of crop plants (Heera, 2002). Anandaraj *et al.* (2003) reported that application of rhizobacteria (*P. fluorescens* and *Bacillus*) and *Trichoderma harzianum* resulted in significantly enhanced growth of black pepper, which resulted in increased number of nodes and cuttings.

Fluorescent pseudomonads are recognized as effective biocontrol agents against most of the fungal and bacterial plant pathogens (Howell and Stipanovic, 1979; Lifshitz *et al.*, 1987; Laha and Verma, 1998; Rangheswaran and Prasad, 2000). They can suppress many plant diseases due to their general biological activities including competition for space and nutrients (Rosales *et al.*, 1995), production of volatile and antimicrobial substances such as iron chelating siderophores and HCN (Raaska *et al.*, 1993; Dave and Dube, 2000; Mondal *et al.*, 2000), inhibition of ethylene synthesis (Glick *et al.*, 1994), production of antibiotics (Schinder *et al.*, 1994; Anith *et al.*, 1999), mobilization of P (Defreitas *et al.*, 1997) and production of growth stimulating phytohormones (Arshad and Frankenberger, 1998). Besides suppression of plant diseases by these mechanisms, fluorescent pseudomonads induce systemic resistance in plants against attack by a wide range of pathogens (Ramamoorthy *et al.*, 2001; Viswanathan and Samiyappan, 2002). *Pseudomonas fluorescence* isolate Pf_1 inhibited mycelial growth of *Pythium aphanidermatum* and promoted plant growth in tomato and chilly due to higher accumulation of Phenylalanine Ammonia Lyase, Peroxidase and Polyphenol Oxidase in the treated plants (Ramamoorthy *et al.*, 2002). Meenakumari *et al.* (2003) also reported that fluorescent pseudomonad isolate P_1 is effective for controlling bacterial wilt of chilli caused by *Ralstonia solanacearum*.

2.9 FLUORESCENT PSEUDOMONADS IN TISSUE CULTURE

Not much work has been done so far to make use of fluorescent pseudomonads in micro propagation. Commercial mycorrhizal inoculants and *P. fluorescence* isolates CHA0 and IP10 have been shown to promote growth during and after weaning stage of potato micro plants (Duffy *et al.*, 1999).

2.10 BIOLOGICAL CONTROL OF FOOT ROT DISEASE OF BLACK PEPPER

Foot rot or quick wilt disease caused by *Phytophthora capsici* Leonian is considered as one of the major reasons for low productivity of black pepper in Kerala (Samraj and Jose, 1966). This disease affects all stages of the crop right from the nursery to the main field. The efficacy of currently used agrochemicals is variable and often not cost effective and undesirable in the health and environmental point of view. Presently microbial antagonists are increasingly being used for the management of the disease (Sarma *et al.*, 1996; Sivaprasad, 1998).

The beneficial effect of arbuscular mycorrhizal association on growth and development of black pepper were reported by many workers (Manjunath and Bagyaraj, 1982; Bopaiah and Khader, 1989; Sivaprasad, 1995b; Robert, 1998; Anandaraj and Sarma, 1994a; Divya, 2002). Anandaraj *et al.* (1993) observed that the use of endomycorrhiza as a biocontrol agent significantly reduced foot rot incidence in pepper. The suppressive effect of AMF was due to enhanced root regeneration, nutrient uptake and altered host physiology in mycorrhizal plants (Anandaraj and Sarma, 1994a; 1994b). Sivaprasad *et al.* (1995b) got a negative correlation between foot rot incidence and percentage of mycorrhizal infection and spore count in black pepper mycorrhizosphere. Anandaraj *et al.* (1996) emphasized the need for using native isolates of arbuscular mycorrhiza for reducing the incidence of foot rot in black pepper. AMF inoculated black pepper cuttings when planted in a diseased field there was significant reduction of disease incidence in mycorrhizal plants (16.5 per cent) as compared to control (28.5 per cent) (Dare, 1996). AMF enhanced rooting in black pepper cuttings and compensate root damage by foot rot pathogen (*P.capsici*) (Sarma *et al.*, 1996).

Black pepper plants inoculated with *Glomus monosporum* had no mortality till 40th day and in inoculated plants there was also an increase in biomass production (Robert, 1998; Sivaprasad *et al.*, 2000). A similar result was also reported by Thanuja and Hegde (2001) in pepper after inoculation with *Glomus fasciculatum*.

Fluorescent pseudomonads were effective in checking the growth of *Phytophthora capsici* and suppressing the expression of foot rot symptoms in black pepper under controlled conditions (Sarma *et al.*, 1996). Exhaustive studies were conducted for the control of soil borne diseases using fluorescent pseudomonads and reported effective suppression of many pathogens (Rangeshwaran and Prasad, 2000). Varshney and Chaube (2000) also reported that *P. fluorescens* was more effective in growth promotion and disease suppression when it was applied along with AMF. *Pseudomonas* isolates, PN-015 and PN-026 colonized the planting material of black pepper and offered protection from *P. capsici* induced nursery wilt (Anith *et al.*, 2002). Fluorescent pseudomonad isolates P_1 and P_{14} isolated from rhizosphere soils of black pepper were found to be effective in reducing foot rot incidence and mortality of black pepper plants from 100 per cent recorded in control to 25 and 6.25 and 56.25 and 18.75 per cent respectively and their effect on growth and root development was also significant (Sivaprasad *et al.*, 2003). Foot rot affected black pepper plants treated with fluorescent pseudomonad, *Pseudomonas putida* strain 89B61 showed dual function of disease suppression and growth promotion (Nair, 2003).

Eventhough Azospirillum was reported to suppress damping off disease in chilly (Kavitha, 2001), no work was reported so far against foot rot disease in black pepper.

2.11 INOCULUM PRODUCTION OF AMF

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

inoculum production are soil based inoculum production, soil less substrate based inoculum production, hydroponic and aeroponic culture (Mosse and Thompson, 1984; Jarstfer and Sylvia, 1994). In almost all these techniques, the inoculum gets mixed with pathogenic and saprophytic microorganisms. The quality of AMF inoculum produced is very important in tissue culture use. Several unsuccessful attempts have been made to culture the organism under *in vitro* conditions using different media (Raman and Sambandan, 2000; Raman *et al.*, 2001). Typical AMF infections can be produced in entire plants grown axenically in agar media (Mosse, 1962). Mosse and Hepper (1975) obtained AMF infections in clover root organ cultures grown in modified White's tissue culture medium containing sucrose. Axenic culture of AMF was obtained in plants grown in agar media (Allen *et al.*, 1979), sand culture (St. John *et al.*, 1981) and in flowing solution culture (Mc Donald, 1981). However, attempts made to make use of transformed root culture technique using *Agrobacterium rhizogenes* for the AMF inoculum production under aseptic conditions met with considerable success (Mugnier and Mosse, 1987; Becard and Fortin, 1988; Chabot *et al.*, 1992; Potty, 1998; Khaliq and Bagyaraj, 2000; Potty, 2001; Tiwari and Adholeya, 2002).

2.11.1 Transformed Root Culture Technique for AMF Inoculum Production

The pathogenic condition known as 'hairy root' is caused on the host plant by *Agrobacterium rhizogenes* (Rikes *et al.*,) Conn. (Ark and Thompson, 1961). The bacterium infects the sensitive host plant and stimulates excessive proliferation of roots (Chilton *et al.*, 1982) by transferring root inducing Ri T-DNA to plant (Schenk, 1982). Becard and Fortin (1988) developed an *in vitro* system using Ri T-DNA transformed carrot roots and vesicular arbuscular mycorrhizal fungus, *Gigaspora margarita* to study the initial events of mycorrhiza formation. Mugnier (1987) could obtain typical infections of *Polymyxa betae* and *Plasmodiophora brassicae* in root organ culture of their host; *Beta vulgaris* or brassicae, containing the root inducing transferred DNA of *A. rhizogenes*. Becard and Piche (1990) studied the physiological factors determining AMF formation in non host (Sugar beet) and host (carrot) Ri T-DNA

transformed roots and found that sugar beet roots lack factors that promote mycorrhizal infection. In addition to *G. margarita* and *G. mosseae*, other species of AMF that have been cultured in transformed roots are *G. intraradices* (Chabot *et al*., 1992), *G. gigantea* (Douds and Becard, 1993). Leu *et al*. (1994) obtained *in vitro* sporulation of AMF, *Gigaspora gigantea* and *Glomus mosseae* by dual culture with carrot Ri T-DNA transformed root and tomato root culture. Declerck *et al*. (1996) and St. Arnaud *et al*. (1996) also reported *in vitro* culture of *G. versiforme* and *G. intraradices* in transformed roots. Bi Yin Li *et al*. (1999) studied the morphological characters of AM symbiosis between AMF and transformed Ri T-DNA carrot roots. Karandashov *et al*. (1999) investigated the pre requisites for *in vitro* culture of AMF together with plant hairy roots. Pawloska *et al*. (1999) reported the establishment of monoxenic cultures of *Glomus etunicatum* in association with excised Ri T-DNA transformed carrot roots. Khaliq and Bagyaraj (2000) also succeeded in making use of transformed root culture technique using *A. rhizogenes* for AMF inoculum production under aseptic conditions. Dalpe and Declerck (2002) for the first time, successfully grew a strain of *Acaulospora rehmii in vitro* in Ri T-DNA transformed carrot roots allowing the *in situ* observation of *Acaulospora* spore development and extra radical thin walled hyphal swellings. The method by which root organ cultures (transformed and non transformed) have been obtained, together with the choice of host species, inoculation techniques and culture media and also the considerable impact that *in vitro* root organ cultures have had on studies of AMF morphology, taxonomy and phylogeny was described (Fortin *et al*., 2002). Tiwari and Adholeya (2002) reported successful co culture of two genera of AMF, *Glomus intraradices* and *Gigaspora margarita* under *in vitro* conditions using Ri T-DNA transformed carrot roots. Various stages in the production of hairy roots in a lesser known tuber crop, *Vigna vexillata* using *Agrobacterium rhizogenes* ATCC 15834 strain for culturing mycorrhizal fungi was reported by Pratapchandran and Potty (2004).

3. MATERIALS AND METHODS

The present investigation on integration of microbial inoculant technology with micro propagation of black pepper (*Piper nigrum* L.) and attempt to standardize the production of arbuscular mycorrhizal fungi (AMF) in *Agrobacterium rhizogenes* mediated transformed root culture was done at College of Agriculture, Vellayani during 2002-2004. The materials and methods involved in the investigations are given hereunder.

3.1 EFFECT OF MICROBIAL INOCULANTS ON SURVIVAL AND GROWTH OF MICRO PROPAGATED BLACK PEPPER PLANTLETS

Tissue culture plantlets of black pepper cultivar Panniyur-4 developed in the Tissue Culture Laboratory, Department of Plant Biotechnology, College of Agriculture, Vellayani were used for the entire study. The nodal segments and leaf segments from selected source plants were used as explants. They were surface sterilized and cultured in culture establishment medium (Half strength MS medium supplemented with growth regulator, BAP) (Murashige and Skoog, 1962) (Appendix I). After callus induction the cultures were transferred to multiplication medium (Half strength MS medium with 1 ppm each of BA and IAA) (Murashige and Skoog, 1962), which enhanced the release of axillary buds. For *in vitro* rooting, the cultures were transferred to rooting medium (Half strength MS medium with 1.5 ppm of IBA) (Murashige and Skoog, 1962). These plantlets were used for planting out, when they produced two to three roots in the rooting medium in three weeks period. Microbial inoculants such as native mixed inoculum of AMF containing isolate of *Glomus fasciculatum*, *Glomus monosporum* and *Glomus sp.*, *Azospirillum brasilense* isolate No. AZR 37 and *Pseudomonas fluorescence* isolate No. P1 obtained from the microbiology section, Department of Plant Pathology, College of Agriculture, Vellayani were used for the entire study.

3.1.1 AMF Inoculum Production and Application

Guinea grass (*Panicum maximum* Jacq.) colonized with selected native cultures of AM fungus was grown in sterilized vermiculite- perlite (16:3:1 w/w) soil for four months. The vermiculite- perlite mixture containing mycorrhizal spores, colonized root segments and hyphae served as mycorrhizal inoculum. The chlamydospores of AMF present in the vermiculite-based medium were isolated following wet sieving and decanting method of spore isolation (Gerdemann and Nicolson, 1963). The medium was made into a suspension with sufficient quantity of water. The suspension was then passed through a series of sieves ranging from 1000, 300, 250 and 105 μm kept one below the other in the same order. The spore suspension in the bottom two sieves was collected in a beaker. The count in the suspension was estimated by transferring one ml of the suspension in a nylon mesh (45μm) and scanned under stereomicroscope. The spore suspension was then mixed with vermiculite so as to obtain 50 spores per gram vermiculite as viable count. This served as AMF inoculum along with colonized roots of guinea grass, which were collected separately and made into small bits of 0.5 cm size.

3.1.2 *Azospirillum* Inoculum Preparation and Application

Nitrogen free bromothymol blue medium (NFB) (Baldani and Dobereiner, 1980) (Appendix I) was inoculated with a loop full of 48 hour old *Azospirillum* isolate AZR 37 and incubated at 28 ± 1 °C for seven days. The full-grown culture was mixed with sterilized lignite powder to a moisture content of 35 per cent under aseptic conditions. The formulation had 10^{10} viable cell count per gram and this was used as *Azospirillum* inoculum.

3.1.3 *Pseudomonas* Inoculum Production and Inoculation

King's B broth (King *et al.*, 1954) (Appendix I) was prepared and sterilized and inoculated with a loop full of 24-hour-old *Pseudomonas* fluorescence isolate P-1 and incubated at a temperature of 28 ± 1 °C for seven days. After seven days, the culture was mixed with sterilized talc powder to a moisture content of 30 per cent

Plate 1 Black pepper plantlets developed *in vitro*

Plate 2 Black pepper plantlet planted out in vermiculite media in plastic pot

under aseptic conditions. The preparation had a population of 10^{12} cells per gram as viable count and used as inoculum for the study.

3.1.4 Planting Out of the Black Pepper Plantlets and Inoculation with Microbial Inoculants

The rooted tissue culture plantlets of black pepper developed in the Tissue Culture Laboratory, Department of Biotechnology were taken out from the culture vessel without injury using forceps and washed repeatedly in sterile distilled water in a beaker to remove adhering pieces of tissue culture medium (Plate 1). Small plastic containers of 7.5 x 6 cm size were filled with sterile sand for planting out the plantlets. In treatments requiring AMF inoculation a portion of the planting medium (sterile sand) was removed from the surface of the pot. Then mycorrhizal inoculum containing one gram of vermiculite based mixture and two gram of root bits were placed as a layer over the planting medium over which the plantlets were placed and filled with sterile sand to cover the root region of plantlets. Similarly wherever treatments required *Azospirillum* inoculation a thick suspension of lignite based *Azospirillum* inoculum (*Azospirillum* culture and water in 1:2 ratio (w/v)) was prepared and the roots of plantlets were dipped in the culture slurry for 15 min before planting out. The method followed for *Azospirillum* inoculation was adopted for the inoculation with *Pseudomonas* also. In treatments requiring both *Azospirillum* and *Pseudomonas* inoculation, *Azospirillum* treatment was given first following treatment with *Pseudomonas*. After planting the remaining slurry of the respective cultures was used to drench the root region of the plantlets (Plate 2).

There were eight treatment combinations as given below in the experiment conducted in Completely Randomized Design (CRD) with 12 replications for each treatment.

Treatment combinations

1. AMF alone (MA_0P_0)
2. *Azospirillum* alone (M_0AP_0)

3. *Pseudomonas* alone (M_0A_0P)

4. Control ($M_0A_0P_0$)

5. *Azospirillum+Pseudomonas* (M_0AP)

6. AMF +*Pseudomonas* (MA_0P)

7. AMF+*Azospirillum* (MAP_0)

8. AMF+*Azospirillum+Pseudomonas* (MAP)

The inoculated plantlets were kept in a mist chamber for 30 days and then shifted to a shade house.

Observations on survival rate, growth characters such as shoot length, number of leaves, number of branches and leaf area were recorded for a period of three months during the hardening period.

3.1.4.1 Survival Rate of Plantlets

The survival rate of black pepper plantlets during hardening period was recorded as percent survival at 10 days interval using the following formula

$$\text{Survival percentage} = \frac{\text{Total number of plants survived}}{\text{Total number of plants in each treatment}} \times 100$$

3.1.4.2 Height of Plantlets (mm)

The height from the ground level to the growing tip of each plantlet was measured and recorded at 30 days Interval.

3.1.4.3 Number of Leaves

The total number of fully opened leaves developed per plantlet was counted and the mean value was recorded.

3.1.4.4 Number of Branches

The total number of branches developed per plantlet was counted and the mean value was recorded.

3.1.4.5 Leaf Area (cm^2)

Leaf area was calculated by plotting the leaves on a graph paper and counting the number of unit squares.

3.1.5 Estimation of percentage Mycorrhizal Colonization

The percentage mycorrhizal colonization in the root samples of black pepper 30 days after planting out was estimated following the procedure of Philips and Hayman (1970). The root samples were cleaned free of soil particles, cut into one cm bits and fixed in FAA (Formaldehyde: Acetic acid: Alcohol in 5:5:90 proportion) for one day. The roots were then autoclaved for hydrolyzing with 10 per cent potassium hydroxide solution at 1.02 kg $cm^{-2}$for 15 minutes. The alkalinity of the samples was then neutralized with one per cent hydrochloric acid. Staining was done by steaming the root bits in 0.05 per cent trypan blue solution in lacto phenol reagent (lactic acid- 20 ml, phenol- 20 ml, glycerol- 40 ml and distilled water- 40 ml) and destaining was done by adding lacto phenol. The stained root bits were arranged on a clean slide covered with cover slips and scanned under compound microscope for the presence of mycelium, vesicles and arbuscules of AM fungi. The AMF colonization percentage was calculated from the formula given below.

$$\text{AMF colonization percentage} = \frac{\text{Number of root bits positive for AMF colonization}}{\text{Total number of root bits observed}} \times 100$$

3.2 EFFECT OF MICROBIAL INOCULANTS ON PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERS OF MICRO PROPAGATED BLACK PEPPER PLANTLETS

The plantlets were subjected to studies on physiological characters such as relative water content (RWC), stomatal conductance and leaf temperature. Biochemical characters such as total phenol content, orthodihydroxy phenol content, protein content and total carbohydrate content were also recorded.

3.2.1 Stomatal Conductance

Stomatal resistance was measured directly by using a porometer (Delta T devices-Cambridge-UK) at 15 days interval and the reading was expressed in s cm^{-1}. The stomatal conductance was calculated from the formula

$$\text{Stomatal conductance} = \frac{1}{\text{Stomatal resistance}} \text{ and expressed in cm } s^{-1}$$

3.2.2 Leaf Temperature

Leaf temperature was measured directly using a porometer (Delta T devices-Cambridge-UK) at 15 days interval and the reading was expressed in °C.

3.2.3 Relative Water Content (RWC)

Fresh weight of leaf samples were taken and put in water in watch glass for 3 hours. After that leaf samples were dried using a blotting paper and turgid weight was measured. These samples were then dried in hot air oven set at a temperature of 90-100 °C for two days and dry weight was taken. The RWC was calculated based on the formula,

$$\frac{\text{Fresh weight - Dry weight}}{\text{Turgid weight - Dry weight}} \times 100$$

3.2.4 Estimation of Total Phenol (Bray and Thorpe, 1954)

One gram of leaf sample was ground in 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min, supernatant was saved and residue was extracted with five times the volume of 80 per cent ethanol and centrifuged. The supernatant was saved and evaporated to dryness. The residue was dissolved in 5ml-distilled water. Folin-Ciocalteau reagent (0.5 ml) was added and 2 ml of 20 percent sodium carbonate solution was added to each tube after three minutes. This was mixed thoroughly and the tubes were placed in a boiling water bath for exactly one minute. This was cooled and was measured at 650 nm in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118) against

reagent blank. Standard curve was prepared using different concentrations of catechol and expressed in catechol equivalents as μg g^{-1} leaf tissue on fresh weight basis.

3.2.5 Estimation of Ortho Dihydroxy Phenol (Johnson and Schaal, 1957)

Three grams of leaf sample was boiled for five minutes in 12 ml of 80 per cent ethanol. After cooling, the tissue was ground using a pestle and mortar and the homogenate was centrifuged (Hettich, EBA 12/2 R) at 10,000 rpm for 20 minutes. The supernatant was saved and the residue was re extracted with 80 per cent ethanol and centrifuged again. The supernatant was collected, pooled and the final volume was adjusted to 15 ml with 80 per cent ethanol. One ml of 0.5 N HCl, one ml of Arnon's reagent and two ml of 1N NaOH were added to one ml of the extract and the volume was made up to 25 ml with 80 per cent ethanol. The absorbance was measured at 540 nm in a UV-VIS spectrophotometer (Systronics UV-VIS Spectrophotometer 118). A standard curve was prepared using different concentrations of catechol (30, 40, 50, 60,70 and 80 μg) and ortho dihydroxy phenol content was expressed as catechol equivalents per gram fresh weight of leaf.

3.2.6 Estimation of Total Soluble Protein (Bradford, 1976)

One gram of leaf sample was homogenized in 10 ml, 0.1 M sodium acetate buffer (pH 4.7) and centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was saved for estimation of soluble protein. The reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml distilled water and 5 ml of diluted (5 times) dye solution (Coomassie brilliant blue G250). The absorbance was read at 595 nm in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118) against reagent blank. Bovine serum albumin was used as the protein standard. The protein content was expressed as μg albumin equivalent of soluble protein per gram on fresh weight basis.

3.2.7 Estimation of Total Carbohydrate (Hedge and Hofreiter, 1962)

Total carbohydrate content was estimated by Anthrone method. Samples of 100 mg each were weighed out and hydrolyzed with five ml of 2.5 N hydrochloric acid (HCl) at 100 °C in a water bath. The hydrolyzate was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged at 5000 rpm for 15 minutes. From the supernatant 0.5 ml aliquot was taken and made up to 1 ml by adding distilled water. To this four ml of anthrone reagent was added and heated for eight minutes at 100 °C in a water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118). Amount of carbohydrate was calculated from standard graph prepared using glucose and expressed in terms of mg of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.3 TOLERANCE TO FOOT ROT PATHOGEN

Another experiment was set up as described in the experiment 3.1 to study the effect of AMF, *Azospirillum* and *Pseudomonas* on suppression of foot rot disease incited by *Phytophthora capsici* on 60 days after planting out. The plantlets were planted out in polythene bags of size 37x25 cm filled with potting mixture containing sand, soil and FYM in the ratio 1:2:0.5. The inoculum preparation and inoculation of microbial inoculants to plantlets were done as described in experiment 3.1. Artificial inoculation with *P. capsici* was done to incite the disease. The per cent disease incidence and mortality were recorded on 10, 20, 30, 40, 50 and 60 th day of inoculation with pathogen in order to assess the disease tolerance if any, due to bio inoculant colonization .The eight treatment combinations in experiment 3.1 was included in the study conducted in CRD with 12 replications.

3.3.1 Isolation of Pathogen

The pathogen inciting foot rot disease in black pepper was isolated from infected black pepper plants collected from College of Agriculture, Vellayani.

Small pieces of infected leaf and vine were initially surface sterilized with 0.1 percent mercuric chloride solution and repeatedly washed with sterile water. These were aseptically transferred to sterile carrot agar medium in petridishes and then incubated at room temperature for three days. Visible growth of mycelium from infected leaf and vine bits were aseptically transferred to sterile slants of carrot agar medium and maintained in a refrigerator for further studies.

3.3.2 Mass Multiplication and Inoculation of Pathogen

The pathogen was mass multiplied in sand oatmeal (19:1) medium.

3.3.2.1 Sand Oatmeal Medium

The medium was prepared by mixing washed fine white sand with oatmeal in the ratio 19:1 (w/w). Sufficient quantity of water was sprinkled and mixed thoroughly to keep the medium moist. The 250 g mixture was sterilized in one litre conical flask at 121 °C for two hours. Actively growing culture bits of the pathogen were aseptically transferred into the flask and incubated at 25- 30 °C for 15 days. The culture so multiplied was used for inoculation in the experiment under investigation.

3.3.2.2 Inoculation of the Pathogen

Inoculation of the pathogen was done by placing sand oatmeal based inoculum in the root region of black pepper plantlets at the rate of 10 g per polythene bag. Each plantlet was inoculated and the roots were again covered with soil. The plants were irrigated regularly so as to maintain a very high humidity for providing conducive environment for the development of the disease and percent disease incidence and mortality was recorded.

3.3.3 Assessment of Foot Rot Infection and Mortality

Plantlets were monitored regularly for foot rot disease incidence and mortality after inoculation with pathogen. The total number of pepper plants

infected and dead were recorded separately on 10,20,30,40,50 and 60 th day of inoculation with pathogen and percentage was computed using the following formula.

$$\text{Infection (Mortality) \%} = \frac{\text{Total no: of plants infected (dead)}}{\text{Total no: of plants in each treatment)}} \times 100$$

3.4 STANDARDIZATION OF *AGROBACTERIUM RHIZOGENES*-TRANSFORMED ROOT CULTURE FOR AMF PRODUCTION

Host plants such as Cucumber *(Cucumis sativus)*, Stylosanthes *(Stylosanthes sps.)*, Pigeon pea *(Cajanus cajan)* tomato (*Lycopersicon esculentum*) and Black pepper (*Piper nigrum*) were screened for studying the sensitivity to *Agrobacterium rhizogenes* infection and root formation. Seeds of cucumber, pigeon pea, tomato, stylosanthes and stem bits containing nodes of black pepper were used for inoculation. The seeds and stem bits of selected host plants were surface sterilized in 0.1% mercuric chloride and placed in MS medium (Murashige and Skoog, 1962) (Appendix I) for germination at 25 °C for seven days. *A. rhizogenes* strain obtained from CTCRI, Sreekaryam, was used for induction of hairy roots. The bacterial culture was preserved in YEA (Yeast Extract Agar) (Appendix I) slants and maintained at 4 °C for the studies. *A.rhizogenes* strain was grown in YEA medium (Ratnasamy, 1997) in culture plates and incubated at 24°C for 24 to 48 hours and was used for inducing infection. *A. rhizogenes* mediated transformation was done by the method described by Ooms *et al.* (1985). Small cuts were made on the stem portion of aseptically grown seedlings/stem bits in MS medium using a sterile scalpel, which was touched with *A. rhizogenes*. The hairy root initiation in the area up to one centimeter above the point of inoculation in the stem was considered as indication of transformation of the seedlings/stem bits. The host plant was selected based on the number and length of roots formed after 24, 48 and 72 hours after inoculation. The transformed seedlings/stem bits were frequently sub cultured in fresh MS media containing antibiotics cefotaxime (250 milligram per litre) and ampicillin (250 milligram per litre) to make the hairy

roots free from *A. rhizogenes*. The bacteria free hairy roots were used for further studies. The hairy roots thus formed through transformation were sub cultured and maintained in fresh rooting medium (Half strength MS with rooting hormone. IBA) (Murashige and Skoog, 1962). Such roots were inoculated with AMF and AMF colonization and proliferation was monitored. For inoculating AMF in transformed roots, the bacteria free plants in petridishes were planted out under aseptic conditions using several media such as sterilized vermiculite, soil rite, sand, vermiculite-perlite mix and vermiculite-sand mix. The plants thus established were inoculated with surface sterilized spores of *G. monosporum*. The hairy roots were also separated from transformed plants and cultured in rooting medium and inoculated with surface sterilized spores of *G. monosporum*.

RESULTS

4. RESULTS

Experiments were conducted to study the effect of inoculation with different beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF), *Pseudomonas fluorescens* and *Azospirillum* on survival, growth, establishment and foot rot incidence and intensity of tissue culture plantlets of black pepper in the hardening phase. Study was also conducted to identify appropriate host plant for transformation with *Agrobacterium rhizogenes* for developing root culture to multiply arbuscular mycorrhizal fungi. The results of various studies are presented here.

4.1 EFFECT OF MICROBIAL INOCULANTS ON SURVIVAL AND GROWTH OF MICROPROPAGATED BLACK PEPPER PLANTS

Inoculation with AMF, *Azospirillum* and *P. fluorescens* has influenced the survival rate and plant growth characteristics such as plant height, number of leaves, number of branches and leaf area of tissue culture plantlets of black pepper.

4.1.1 Effect of Microbial Inoculants on Survival of Tissue Culture Plantlets of Black Pepper

The survival rate of the plantlets was found to improve due to inoculation with microbial inoculants (Table 1)(Fig.1). There was not much mortality of plantlets till 20 days after transplanting (DAT). Considerable mortality was noticed from 30th day of transplanting in control (33.3 per cent mortality) and *Azospirillum* alone (25.0 per cent mortality) (Plate 3). The treatments M_0A_0P, M_0AP and MAP_0 recorded 100.0 per cent survival, whereas mycorrhiza alone, MA_0P and MAP showed 91.6 per cent each. On 40th day also M_0A_0P, M_0AP and MAP_0 recorded 100.0 per cent survival. The control and *Azospirillum* alone recorded 66.6 per cent each. Mycorrhiza alone and that with *Pseudomonas*

Table 1. Effect of microbial inoculants on survival of tissue culture plantlets of black pepper

Treatments	Number of plants survived					
	20 DAT	30 DAT	40 DAT	50 DAT	60 DAT	90 DAT
MA_0P_0	11 (91.6)	11 (91.6)	10 (83.3)	10 (83.3)	10 (83.3)	10 (83.3)
M_0AP_0	11 (91.6)	9 (75)	8 (66.6)	8 (66.6)	8 (66.6)	8 (66.6)
M_0A_0P	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)
$M_0A_0P_0$	12 (100)	8 (66.6)	8 (66.6)	8 (66.6)	8 (66.6)	8 (66.6)
M_0AP	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)
MA_0P	12 (100)	11 (91.6)	11 (91.6)	11 (91.6)	10 (83.3)	10 (83.3)
MAP_0	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)
MAP	11 (91.6)	11 (91.6)	11 (91.6)	11 (91.6)	11 (91.6)	11 (91.6)

Figures in parentheses are values in percentage

Table 2. Effect of microbial inoculants on height (mm) of tissue culture plantlets of black pepper

Treatments	30 DAT	60 DAT	90 DAT
MA_0P_0	35.00	39.14	60.00
M_0AP_0	28.33	34.47	55.00
M_0A_0P	30.00	34.28	46.66
$M_0A_0P_0$	26.66	28.80	39.66
M_0AP	29.00	33.71	48.33
MA_0P	41.00	48.85	64.66
MAP_0	32.66	37.23	56.66
MAP	39.33	46.33	69.00
CD (0.05)	NS	15.48	13.84

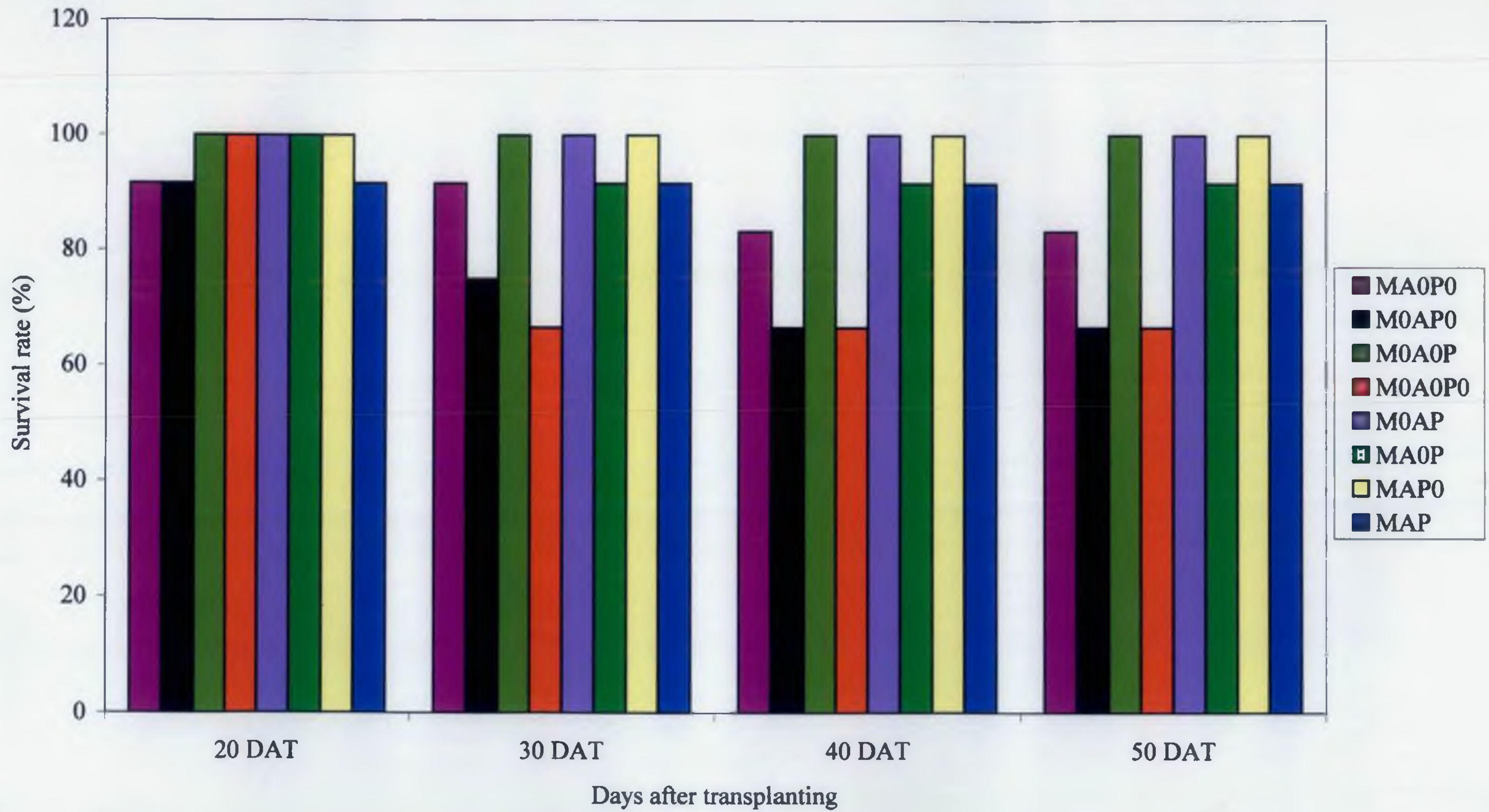


Fig. 1 Effect of microbial inoculants on survival of tissue culture plantlets of black pepper

A - Inoculated with *Azospirillum* C - Control

Plate 3 Black pepper plantlets inoculated with *Azospirillum* at the time of planting out

P - Inoculated with *Pseudomonas* C - Control

Plate 4 Black pepper plantlets inoculated with *Pseudomonas* at the time of planting out

M - Inoculated with AMF C - Control

Plate 5 Black pepper plantlets inoculated with mycorrhiza at the time of planting out

M - Inoculated with AMF C - Control

Plate 6 Growth of tissue culture plantlets of black pepper inoculated with mycorrhiza at the time of planting out

P - Inoculated with *Pseudomonas* C - Control

Plate 7 Growth of tissue culture plantlets of black pepper inoculated with *Pseudomonas* at the time of planting out

A - Inoculated with *Azospirillum* C - Control

Plate 8 Growth of tissue culture plantlets of black pepper inoculated with *Azospirillum* at the time of planting out

recorded 83.3 and 91.6 per cent survival. There was no further mortality, irrespective of the treatment, till 90th day of observation. The treatment involving *Pseudomonas* and mycorrhiza either alone or in combination consistently showed remarkably higher survival rate compared to control and *Azospirillum* alone inoculation (Plate 4) (Plate 5).

4.1.2 Effect of Microbial Inoculants on Height (mm) of Tissue Culture Plantlets of Black Pepper

The observations recorded on plant height at 30th, 60th and 90th day of planting out after giving inoculation with various microbial inoculants were presented in Table 2. Amongst the individual treatments, mycorrhiza (MA_0P_0) recorded the maximum plant height of 35.00, 39.14 and 60.00 mm (Plate 6) while *Pseudomonas* (M_0A_0P) recorded plant height of 30.00, 34.28 and 46.66 mm (Plate 7) and *Azospirillum* showed plant height of 28.33, 34.47 and 55.00 mm (Plate 8) on 30th, 60th and 90th day of planting out respectively. These treatments were found to be significantly superior over control, which recorded 26.66, 28.80 and 39.66 mm on 30th, 60th and 90th day respectively (Table 2). The combined inoculation of microbial inoculants showed further increase in plant height at various stages of observation. Treatment, M_0AP recorded plant height of 48.33 mm on 90th day, which was superior over *Pseudomonas* though, less than *Azospirillum*. The combination treatment of AMF and *Pseudomonas* consistently showed higher plant height compared to their individual inoculations and control treatment. The treatment MA_0P recorded plant height of 41.00, 48.85 and 64.66 mm, as against 35.00, 39.14 and 60.00 mm noticed in the case of mycorrhiza alone and 30.00, 34.28 and 46.66 mm in *Pseudomonas* alone on 30th, 60th and 90th day of planting out respectively. The dual inoculation of *Azospirillum* and AMF did not show much effect over their individual inoculations. The combination treatment MAP was found to be most effective which recorded maximum plant height of 69.00 mm on 90th day of planting out (Table 2). This treatment

Table 3. Effect of inoculation of microbial inoculants on leaf formation in tissue culture plantlets of black pepper

Treatments	30 DAT	60 DAT	90 DAT
MA_0P_0	4.66	7.33	7.66
M_0AP_0	5.33	6.66	7.33
M_0A_0P	5.00	7.00	8.00
$M_0A_0P_0$	3.66	6.00	6.00
M_0AP	5.33	7.33	7.66
MA_0P	5.66	8.00	8.66
MAP_0	6.00	7.33	8.33
MAP	5.66	8.66	9.33
CD (0.05)	NS	NS	NS

Table 4. Effect of AMF, *Azospirillum*, *Pseudomonas* on number of branches of tissue culture plantlets of black pepper

Treatments	30 DAT	60 DAT	90 DAT
MA_0P_0	1.66	2.00	2.00
M_0AP_0	1.33	1.66	1.66
M_0A_0P	2.00	2.33	2.33
$M_0A_0P_0$	1.33	1.66	1.66
M_0AP	2.00	2.33	2.33
MA_0P	1.66	2.00	2.33
MAP_0	2.00	2.33	2.66
MAP	2.00	2.66	3.00
CD (0.05)	NS	NS	NS

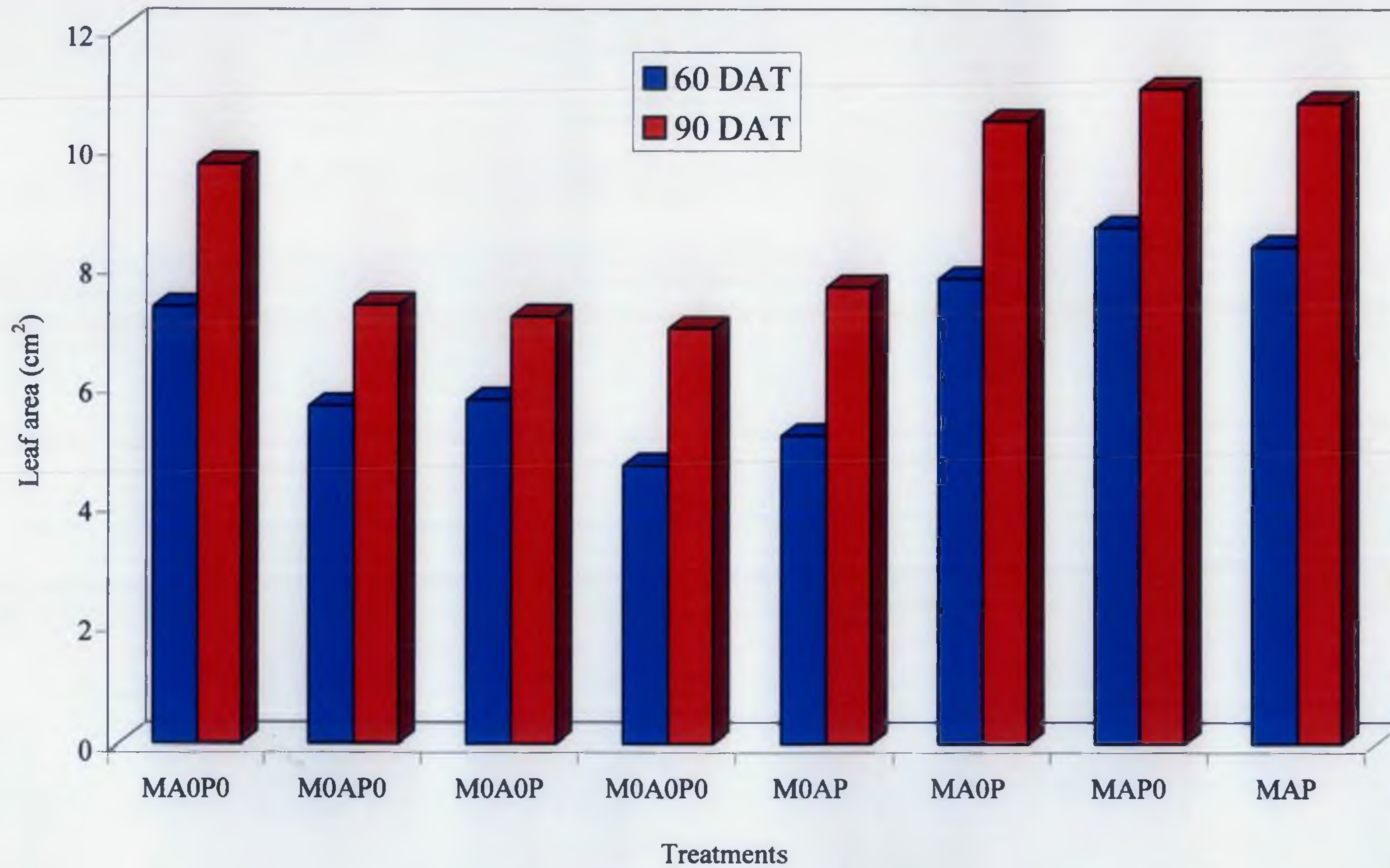


Fig. 2 Leaf area (cm^2) of tissue culture plantlets of black pepper as influenced by inoculation with microbial inoculants

was significant over control (39.66 mm), individual inoculation of *Azospirillum* (55.00 mm), *Pseudomonas* (46.66 mm) and dual inoculation of *Azospirillum* and *Pseudomonas* (48.33 mm), though statistically on par with MA_0P (64.66 mm) and MA_0P_0 (60.00 mm).

4.1.3 Effect of Inoculation of Microbial Inoculants on Leaf Formation in Tissue Culture Plantlets of Black Pepper

There was no significant difference in number of leaves between the treatments on 30th, 60th and 90th day of observation (Table 3). However an increasing trend in number of leaves due to various treatments was noticed. The maximum number of leaves was shown by treatment MAP on 60th (8.66) and 90th (9.33) day of planting out, whereas the control recorded the lowest number of leaves (6.00).

4.1.4 Effect of AMF, *Azospirillum*, *Pseudomonas* on Number of Branches of Tissue Culture Plantlets of Black Pepper

There was no significant difference between the treatments in number of branches at all stages of observation. The maximum number of 3 branches was recorded in MAP on 90th day of planting out as against 1.66 of control (Table 4).

4.1.5 Leaf Area (cm^2) of Tissue Culture Plantlets of Black Pepper as Influenced by Inoculation with Microbial Inoculants

A general increase in leaf area of the plantlets noticed due to inoculation with microbial inoculants (Table 5) (Fig.2). All the treatments involving AMF showed higher leaf area compared to other treatments. Amongst treatments MA_0P_0, M_0AP_0 and M_0A_0P, maximum leaf area of 5.43, 7.33 and 9.73 cm^2 on 30, 60 and 90 DAT was shown by MA_0P_0, while M_0AP_0 recorded leaf area of 3.60, 5.66 and 7.36 cm^2 and M_0A_0P showed leaf area of 3.53, 5.76 and 7.16 cm^2 respectively. These treatments were found to be superior over control, which recorded leaf area of 3.23, 4.66 and 6.96 cm^2 respectively (Table 5). Among the dual inoculation

treatments, M_0AP did not show much effect over their individual inoculations, M_0AP_0 and M_0A_0P. The combined application of AMF and *Pseudomonas* recorded a higher leaf area of 10.46 cm^2 (Plate 9) on 90 DAT which was significant over control (6.96 cm^2) and M_0A_0P (7.16 cm^2). However, it was on par with MA_0P_0 (9.73 cm^2). The dual application of *Azospirillum* and AMF recorded maximum leaf area of 11.00 cm^2 (Plate 10) on 90th day of planting out which was significant over control (6.96 cm^2) and M_0AP_0 (7.36 cm^2) (Table 5). The combination treatment MAP showed a leaf area of 10.76 cm^2 (Plate 11) on 90th day, which was on par with MA_0P_0 (9.73 cm^2), MA_0P (10.46 cm^2) and MAP_0 (11.00 cm^2).

4.1.6 Mycorrhizal Colonization (30 DAT) in Tissue Culture Plantlets of Black Pepper Inoculated with Microbial Inoculants (%)

Mycorrhizal colonization in the plantlets observed on 30 DAT indicated a higher colonization percentage ranging from 50.00 to 66.70 in treatments, which received mycorrhizal inoculation (Table 6) (Plate 12). There was no colonization in uninoculated treatments. Highest colonization of 66.70 per cent was recorded in MA_0P_0, followed by MA_0P with 54.00 per cent, MAP_0 with 53.30 per cent and MAP with 50.00 per cent.

4.2 EFFECT OF MICROBIAL INOCULANTS ON PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF MICROPROPAGATED BLACK PEPPER PLANTLETS

4.2.1 Influence of Microbial Inoculants on Stomatal Conductance (cm s^{-1}) of Tissue Culture Plantlets of Black Pepper

A considerable reduction in stomatal conductance in all the plantlets due to inoculation with microbial inoculants was noticed on 15, 30 and 45 DAT (Table 7) (Fig.3). On 15 DAT, a general reduction in stomatal conductance was noticed in all the treatments when compared to control with 0.53 cm s^{-1}. Although, there was no significant difference

Table 5. Leaf area (cm^2) of tissue culture plantlets of black pepper as influenced by inoculation with microbial inoculants

Treatments	30 DAT	60 DAT	90 DAT
MA_0P_0	5.43	7.33	9.73
M_0AP_0	3.60	5.66	7.36
M_0A_0P	3.53	5.76	7.16
$M_0A_0P_0$	3.23	4.66	6.96
M_0AP	3.53	5.16	7.66
MA_0P	5.70	7.80	10.46
MAP_0	4.90	8.66	11.00
MAP	7.66	8.33	10.76
CD (0.05)	1.19	1.08	1.31

Table 6. Mycorrhizal colonization (30 DAT) in tissue culture plantlets of black pepper inoculated with AMF, *Azospirillum* and *Pseudomonas* (%)

Treatments	Colonization percentage
MA_0P_0	66.70
M_0AP_0	0.00
M_0A_0P	0.00
$M_0A_0P_0$	0.00
M_0AP	0.00
MA_0P	54.00
MAP_0	53.30
MAP	50.00

A+P - Inoculated with *Azospirillum* and *Pseudomonas*, C - Control

Plate 9 Growth of tissue culture plantlets of black pepper inoculated with *Azospirillum* and *Pseudomonas* at the time of planting out

M+P - Inoculated with AMF and *Pseudomonas*, C - Control

Plate 10 Growth of tissue culture plantlets of black pepper inoculated with AMF and *Pseudomonas* at the time of planting out

A+M - Inoculated with *Azospirillum* and AMF, C - Control

Plate 11 Growth of tissue culture plantlets of black pepper inoculated with *Azospirillum* and mycorrhiza at the time of planting out

A+M+P - Inoculated with *Azospirillum*, AMF and *Pseudomonas*, C - Control

Plate 12 Growth of tissue culture plantlets of black pepper inoculated with *Azospirillum*, mycorrhiza and *Pseudomonas* at the time of planting out

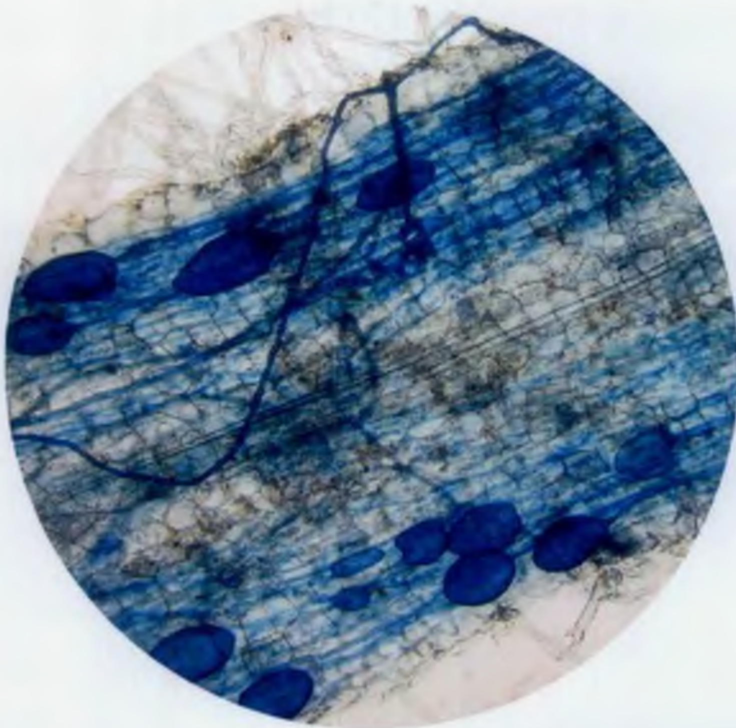


Plate 13 Mycorrhizal colonization in tissue culture plantlets of black pepper at 30 days after transplanting

Table 7. Influence of microbial inoculants on stomatal conductance (cm s^{-1}) of tissue culture plantlets of black pepper

Treatments	15 DAT	30 DAT	45 DAT
MA_0P_0	0.41	0.18	0.17
M_0AP_0	0.42	0.12	0.15
M_0A_0P	0.41	0.14	0.16
$M_0A_0P_0$	0.53	0.23	0.13
M_0AP	0.46	0.19	0.17
MA_0P	0.33	0.15	0.16
MAP_0	0.43	0.18	0.17
MAP	0.49	0.12	0.18
CD (0.05)	NS	0.06	NS

Table 8. Effect of microbial inoculants on leaf temperature (°C) of tissue culture plantlets of black pepper

Treatments	15 DAT	30 DAT	45 DAT
MA_0P_0	33.20	32.30	33.40
M_0AP_0	33.30	33.43	33.93
M_0A_0P	33.50	33.60	32.70
$M_0A_0P_0$	33.47	35.36	35.63
M_0AP	33.17	34.13	33.50
MA_0P	33.47	33.00	32.26
MAP_0	33.03	32.73	32.43
MAP	33.37	32.83	32.30
CD (0.05)	NS	0.41	0.44

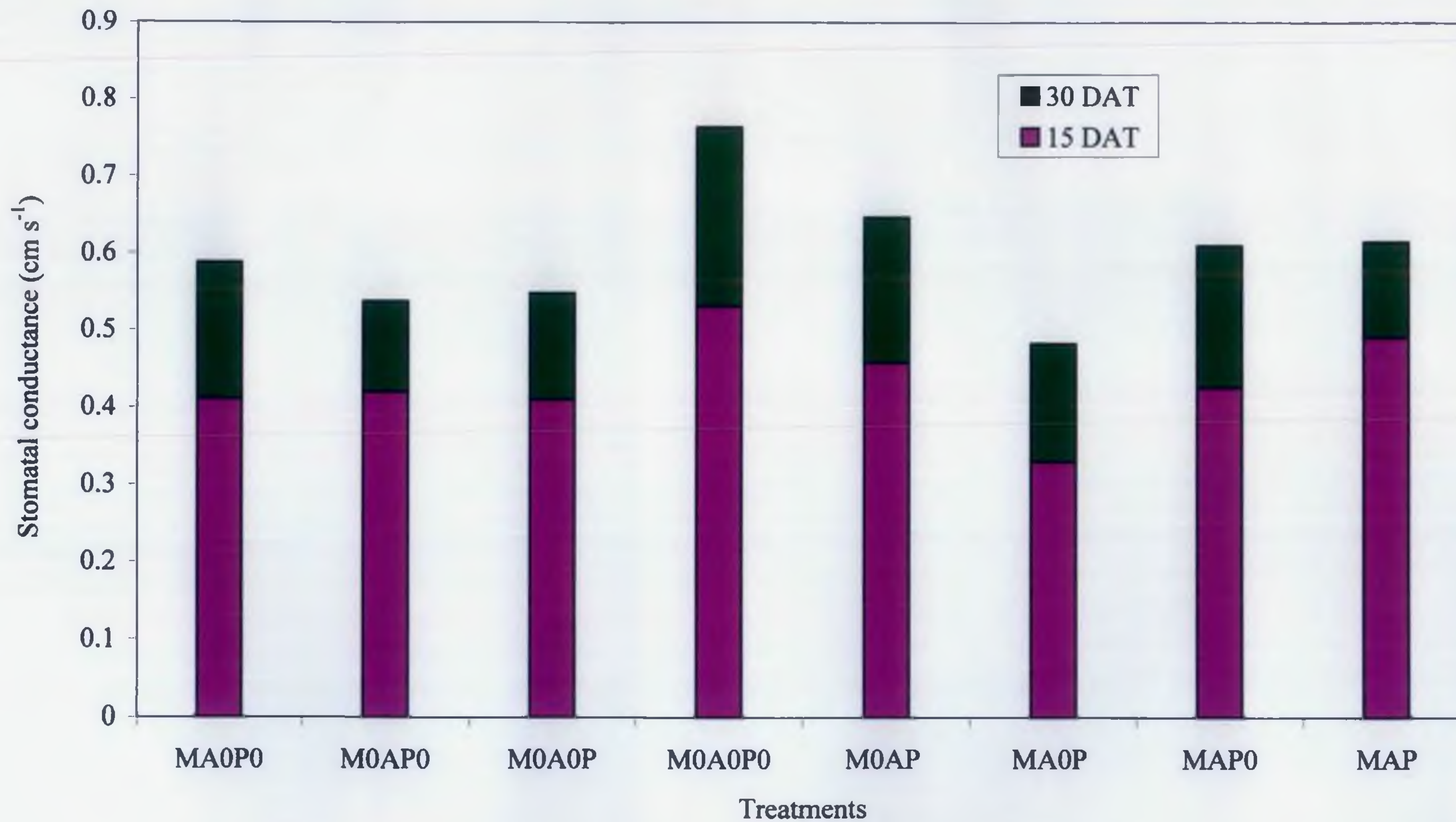


Fig. 3 Influence of microbial inoculants on stomatal conductance (cm s^{-1}) of tissue culture plantlets of black pepper

between the treatments, the lowest stomatal conductance was recorded by the treatment MA_0P (0.33 cm s^{-1}) (Table 7). By 30th day of transplanting, stomatal conductance was found to decrease in all the treatments. The lowest stomatal conductance was recorded in treatment M_0AP_0 (0.12 cm s^{-1}) which was on par with MA_0P_0 (0.18 cm s^{-1}) and M_0A_0P (0.14 cm s^{-1}) and also combination of treatments such as MA_0P (0.15 cm s^{-1}) and MAP (0.12 cm s^{-1}). The control treatment recorded a higher stomatal conductance of 0.23 cm s^{-1} on 30 DAT also. It was observed that the individual inoculation treatments recorded lower values of stomatal conductance than combination treatments (Table 7). The observations taken on 45 DAT showed no significant difference among the various treatments. Stomatal conductance was found to increase in tissue culture plantlets due to inoculation with different microorganisms (Table 7). All the treatments involving mycorrhiza consistently showed higher values for stomatal conductance. Maximum stomatal conductance of 0.18 cm s^{-1} was recorded in MAP followed by MA_0P_0 and MAP_0 with 0.17 cm s^{-1} each. The control recorded 0.13 cm s^{-1}

4.2.2 Effect of Microbial Inoculants on Leaf Temperature (°C) of Tissue Culture Plantlets of Black Pepper

The leaf temperature of the plantlets was recorded at 15 days interval for 45 days and the data presented in Table 8. No significant difference in leaf temperature was noticed at 15 DAT among the various treatments. The observations made on 30 DAT showed a significantly lower leaf temperature in plantlets inoculated with microbial inoculants when compared to uninoculated control. There was a definite decrease in leaf temperature in treatments involving mycorrhizal inoculation. MA_0P_0 showed lowest leaf temperature of 32.30°C, while treatments M_0AP_0 and M_0A_0P recorded leaf temperature of 33.43°C and 33.60°C respectively, which were significantly less than that of control plantlets, which recorded a leaf temperature of 35.36°C (Table 8). Among dual inoculation

treatments, lowest temperature was shown by MAP_0 (32.73°C) which was less than M_0AP_0 (33.40°C). The AMF alone treatment (MA_0P_0) recorded 32.30°C. The treatment MA_0P recorded a leaf temperature of 33.00°C, which was lower than *Pseudomonas* alone (33.60°C). The leaf temperature recorded for M_0AP was 34.13°C, which was significantly less than that of control (35.36°C). The treatment involving all the organisms showed the lowest leaf temperature of 32.83°C. All these treatments were found significantly superior over control treatment (Table 8). On 45 DAT also the plantlets inoculated with different microbial inoculants showed significantly lower leaf temperature than uninoculated control (Table 7). Among individual inoculation treatments, *Pseudomonas* showed lowest leaf temperature of 32.70°C, while Azospirillum and AMF recorded 33.93°C and 33.40°C respectively. Amongst all the treatments, the lowest temperature was recorded by MA_0P (32.26°C), which was on par with MAP (32.30°C). The treatment MAP_0 also recorded a lower leaf temperature of 32.43°C.

4.2.3 Effect of Microbial Inoculants on Relative Water Content (RWC) (%) of Tissue Culture Plantlets of Black Pepper

RWC observed on 60, 120 and 180 DAT showed an increasing trend due to inoculation with various microbial inoculants when compared to uninoculated control. The data is presented in Table 9 (Fig.4). Although no significant difference in RWC was noticed among various treatments at 60 DAT, the highest value of 79.36 per cent was shown by MAP as against 65.30 per cent noticed in control treatment. The treatments involving individual inoculations of the three microbial inoculants *viz.*, AMF, *Azospirillum* and *Pseudomonas* was found to show an increasing trend in RWC. At 120 DAT, plantlets which received individual inoculations with *Azospirillum*, AMF and *Pseudomonas* showed significantly higher RWC over control (Table 9). Of the three treatments, RWC was maximum for M_0AP_0 (83.40 %), which was on par with MA_0P_0

Table 9. Effect of microbial inoculants on relative water content (RWC) (%) of tissue culture plantlets of black pepper

Treatments	60 DAT	120 DAT	180 DAT
MA_0P_0	73.93	80.90	86.33
M_0AP_0	75.13	83.40	94.70
M_0A_0P	70.13	80.63	92.56
$M_0A_0P_0$	65.30	70.40	73.80
M_0AP	76.26	79.66	85.46
MA_0P	73.03	77.26	82.20
MAP_0	74.06	80.50	84.40
MAP	79.36	88.60	90.83
CD (0.05)	NS	8.56	7.31

Table 10. Total phenol content (μg g^{-1} leaf tissue) of tissue culture plantlets of black pepper as influenced by microbial inoculants

Treatments	60 DAT	120 DAT
MA_0P_0	74.80	105.83
M_0AP_0	58.50	99.36
M_0A_0P	46.67	89.83
$M_0A_0P_0$	47.83	76.20
M_0AP	55.83	85.00
MA_0P	61.30	97.13
MAP_0	65.83	100.33
MAP	67.33	101.80
CD (0.05)	9.05	16.94

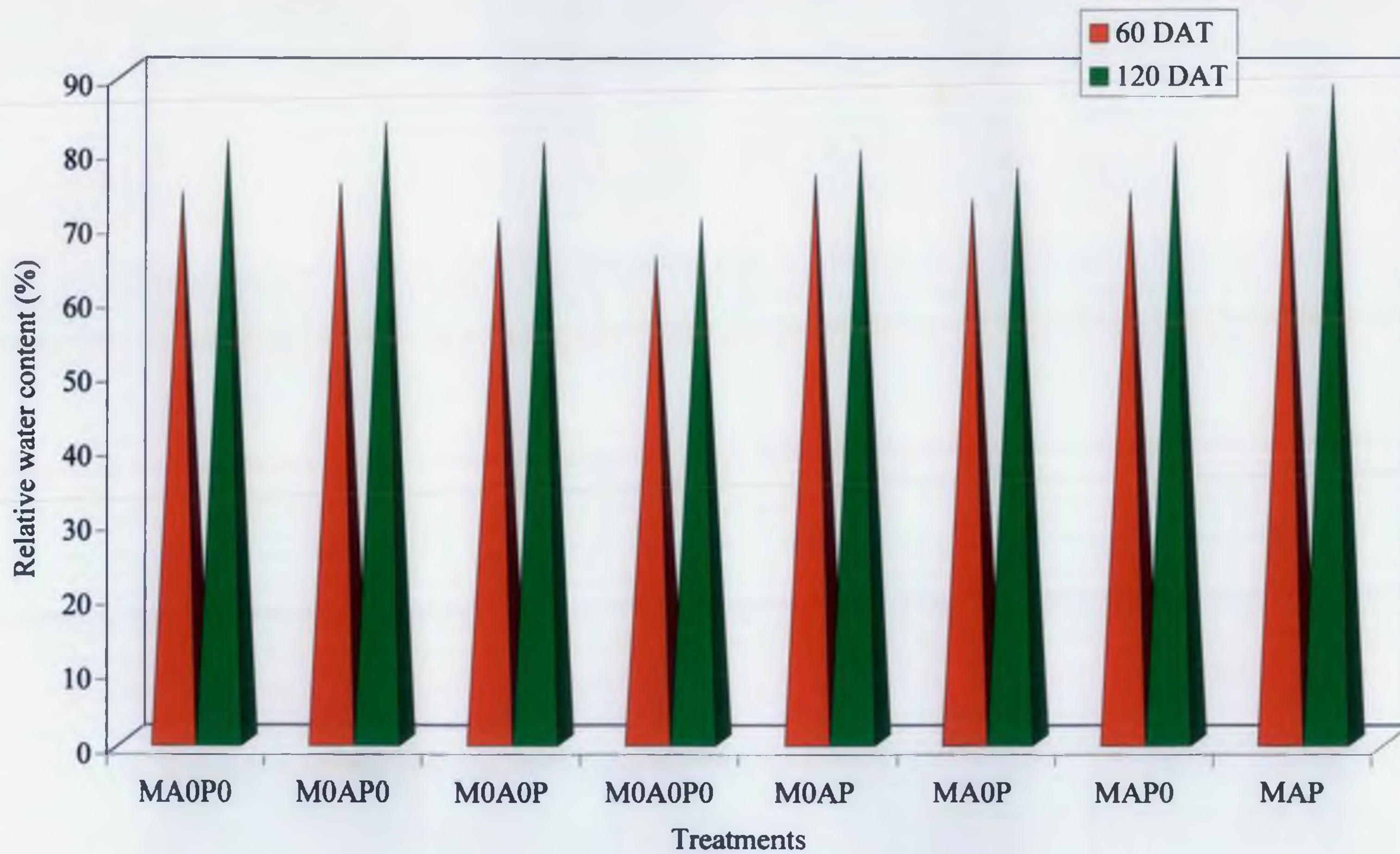


Fig. 4 Effect of microbial inoculants on relative water content (RWC) (%) of tissue culture plantlets of black pepper

(80.90 %) and M_0A_0P (80.63%), though all the three treatments were significantly higher than control (70.40 %). Amongst the dual inoculation treatments, M_0AP, M A_0P and MAP_0, the maximum RWC was shown by MAP_0 (80.50 %), which was on par with M_0AP (79.66 %) and MA_0P (77.26 %). These three treatments did not show significant effect over their individual inoculation treatments MA_0P_0, M_0AP_0 and M_0A_0P. The highest RWC was recorded by treatment MAP (88.60%) which was significantly higher than M_0AP, MA_0P and control and was on par with M_0AP_0, MA_0P_0, M_0A_0P and MAP_0. At 180 DAT also all the treatments involving microbial inoculants recorded significantly higher RWC (Table 9). M_0AP_0 recorded maximum RWC of 94.70 per cent, which was on par with M_0A_0P (92.56%) and MA_0P_0 (86.33%). The combination treatments such as M_0AP (85.46 %), MA_0P (82.20) and MAP_0 (84.40) were statistically on par and did not show significant effect over their individual inoculation treatments. The combined inoculation with *Azospirillum*, AMF and *Pseudomonas* recorded RWC of 90.83 per cent, which was on par with the maximum value of 92.56 per cent recorded, with *Pseudomonas* alone. It was observed that the dual inoculation with microbial inoculants generally showed a relatively less RWC compared to their individual inoculations at all stages of observation. However, the combination involving all the three organisms recorded consistently higher values at different stages of observations.

4.2.4 Total Phenol Content (µg g^{-1} leaf tissue) of Tissue Culture Plantlets of Black Pepper as Influenced by Microbial Inoculants

The total phenol content was recorded at 60 and 120 DAT and found a general increase in total phenol content in treatments containing microbial inoculation with maximum effect being in AMF inoculation (Table 10) (Fig.5). At 60 DAT, maximum phenol content of 74.80 µg g^{-1} was recorded in treatment with mycorrhiza alone (MA_0P_0) while M_0AP_0

and M_0A_0P showed 58.50 and 46.67 μg g^{-1} respectively, as against 47.83 μg g^{-1} observed in control plantlets. Amongst the dual inoculation treatments *viz*., M_0AP, MA_0P and MAP_0, maximum phenol content was shown by MAP_0 (65.83 μg g^{-1}) though lower than MA_0P_0 (74.80 μg g^{-1}). The treatment MA_0P (61.30 μg g^{-1}) was superior over M_0A_0P (46.67 μg g^{-1}), but less than MA_0P_0. The treatment M_0AP (55.83 μg g^{-1}) did not show much effect over their individual inoculations. All the inoculation treatments except M_0A_0P and M_0AP recorded a significant increase in total phenol content in the tissues of plants over control (Table 10). Mycorrhiza alone inoculation recorded maximum phenol content of 105.83 μg g^{-1} amongst all the treatments at 120 DAT. Individual inoculation with *Azospirillum* and *Pseudomonas* recorded values of 99.36 μg g^{-1} and 89.83 μg g^{-1} respectively. The individual inoculations with AMF and *Azospirillum* were superior over control, which recorded 76.20 μg g^{-1}. Amongst the dual inoculation treatments, MAP_0 recorded higher phenol content of 100.33 μg g^{-1} followed by MA_0P with 97.13 μg g^{-1} and M_0AP with 85.00 μg g^{-1}. The treatment combination involving all the three organisms recorded 101.80 μg g^{-1} which was significantly higher than control but on par with other inoculation treatments (Table 10). Amongst the organisms, AMF was found to stimulate maximum total phenol production in plant tissues followed by *Azospirillum* while the effect of *Pseudomonas* was relatively low.

4.2.5 Orthodihydroxy (OD) Phenol Content (μg g^{-1} leaf tissue) of Tissue Culture Plantlets of Black Pepper as Influenced by Microbial Inoculants

Observations taken on 60 and 120 DAT showed that all the inoculation treatments significantly increased the OD phenol content of the plant tissue over control. A general increase in OD phenol content was observed in plants inoculated with AMF and *Pseudomonas* (Table 11) (Fig. 5). At 60 DAT, amongst individual treatments, maximum OD phenol

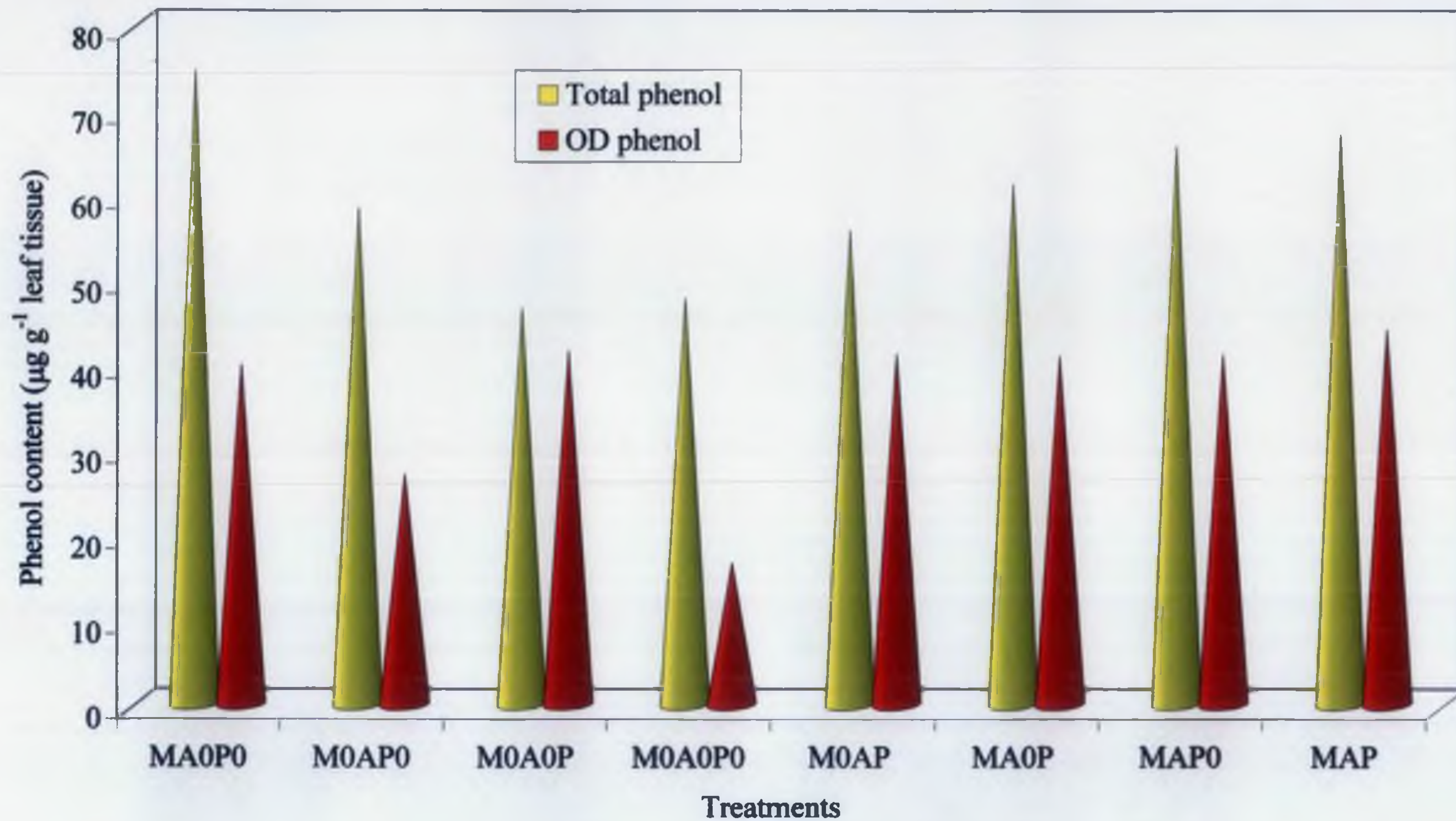


Fig. 5 Total phenol ($\mu g\ g^{-1}$) and orthodihydric (OD) phenol ($\mu g\ g^{-1}$) content of tissue culture plants of black pepper as influenced by microbial inoculants

Table 11. Orthodihydroxy (OD) phenol content (μg g^{-1} leaf tissue) of tissue culture plantlets of black pepper as influenced by microbial inoculants

Treatments	60 DAT	120 DAT
MA_0P_0	40.00	40.76
M_0AP_0	27.16	37.20
M_0A_0P	41.76	43.50
$M_0A_0P_0$	16.66	36.56
M_0AP	41.33	42.73
MA_0P	41.10	40.70
MAP_0	41.26	42.56
MAP	44.16	46.66
CD (0.05)	3.23	4.27

Table 12. Protein content (μg g^{-1} leaf tissue) of tissue culture plantlets of black pepper as influenced by microbial inoculants

Treatments	60 DAT	120 DAT
MA_0P_0	396.6	490.0
M_0AP_0	446.6	496.6
M_0A_0P	456.0	701.6
$M_0A_0P_0$	291.6	351.6
M_0AP	535.0	620.3
MA_0P	468.3	675.0
MAP_0	495.0	656.3
MAP	590.0	720.0
CD (0.05)	72.2	59.9

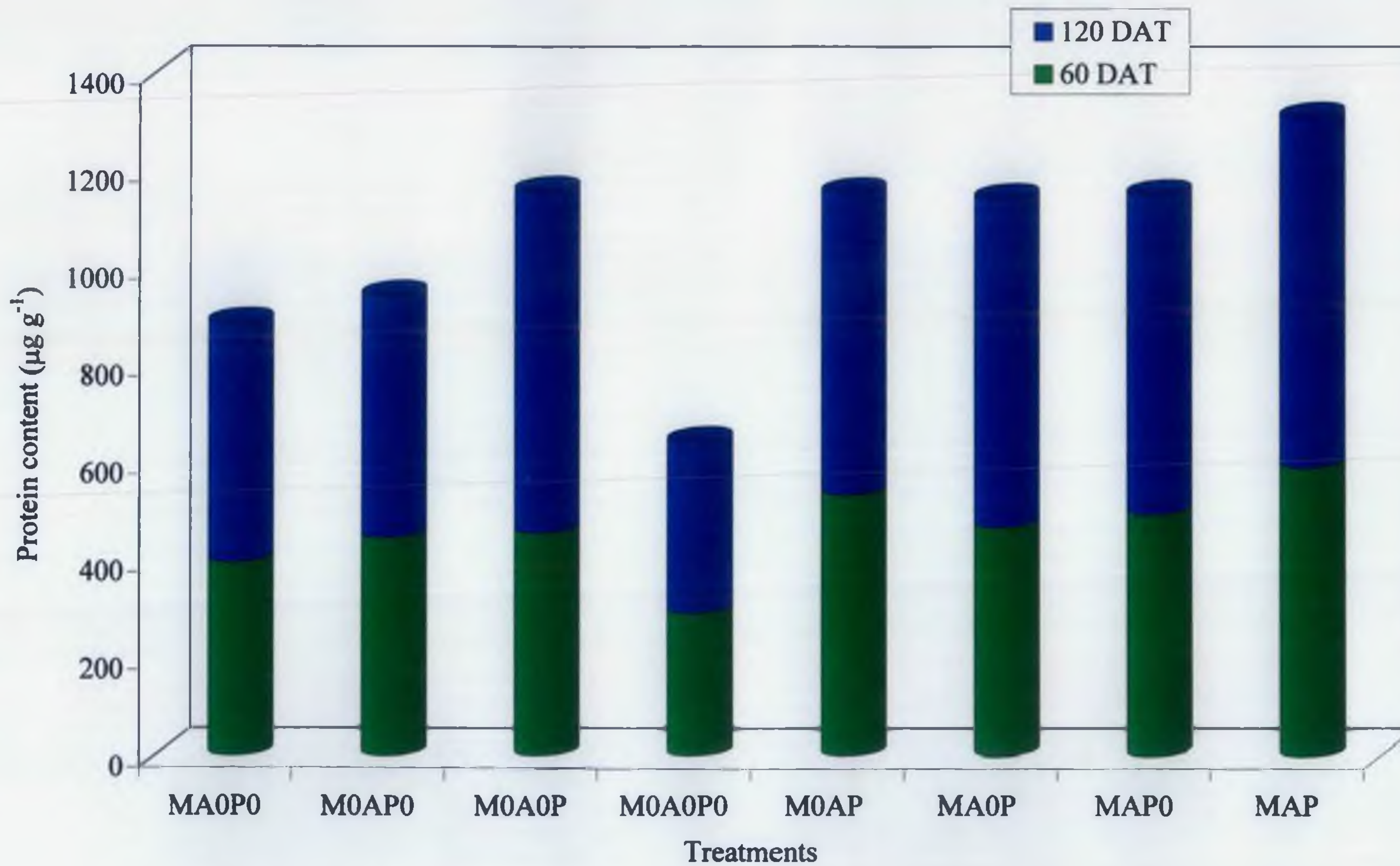


Fig. 6 Protein content (μg g^{-1}) of tissue culture plants of black pepper as influenced inoculation with microbial inoculants

value of 41.76 µg g^{-1} was recorded in treatment with *Pseudomonas* alone followed by AMF with 40.00 µg g^{-1} and *Azospirillum* having 27.16 µg g^{-1}. All these treatments were found to be significantly higher than control treatment, which recorded OD phenol content of 16.66 µg g^{-1}. The dual inoculation treatments such as M_0AP, MA_0P and MAP_0 did not show much variation amongst treatments and their values ranged from 41.10 to 41.33 µg g^{-1}. The highest OD phenol content was recorded with combination of AMF, *Azospirillum* and *Pseudomonas* with 44.16 µg g^{-1} and was on par with dual inoculation treatments and treatment with *Pseudomonas* alone. It was found that the effect of *Azospirillum* inoculation on OD phenol content, though significantly increased (27.16 µg g^{-1}) over control (16.66 µg g^{-1}), was relatively less when compared to AMF and *Pseudomonas* (Table 10). A similar trend was noticed on 120 DAT also. All the inoculation treatments showed significant increase over control (Table 11). OD phenol values recorded for MA_0P_0, M_0AP_0 and M_0A_0P were 40.76, 37.20 and 43.50 µg g^{-1} respectively. The control treatment showed OD phenol content of 36.56 µg g^{-1} leaf tissue. There was not much difference amongst the dual inoculation treatments *viz.*, M_0AP (42.73 µg g^{-1}), MA_0P (40.70 µg g^{-1}) and MAP_0 (42.56 µg g^{-1}). The combination involving all the three microbial inoculants was most effective and recorded the highest OD phenol content of 46.66 µg g^{-1} (Table 11).

4.2.6 Protein Content (µg g^{-1} leaf tissue) of Tissue Culture Plantlets of Black Pepper as Influenced by Microbial Inoculants

The protein content of plant tissue was estimated on 60 and 120 DAT. The observations showed a significant increase in protein content of the plantlets due to inoculation with AMF, *Azospirillum*, *Pseudomonas* and their combinations (Table 12) (Fig. 6). On 60 DAT, amongst treatments MA_0P_0, M_0AP_0 and M_0A_0P, maximum protein content was recorded by M_0A_0P (456.0 µg g^{-1}), followed by MA_0P_0 (396.6 µg g^{-1}) and

M_0AP_0 (446.6 μg g^{-1}). The control plantlets recorded a lower protein content of 291.6 μg g^{-1}. In the dual inoculation treatments, M_0AP recorded maximum protein content of 535.0 μg g^{-1}, which was significantly higher than individual inoculation with *Azospirillum*, *Pseudomonas* and AMF while MA_0P (468.3 μg g^{-1}) did not show considerable effect over mycorrhiza and *Pseudomonas*. Treatment MAP_0 (495.0 μg g^{-1}) showed significant increase in protein content over M_0AP_0 and MA_0P_0. Although AMF recorded relatively low protein content compared to *Pseudomonas* and *Azospirillum*, its combined inoculation with *Azospirillum* (495.0 μg g^{-1}) and *Pseudomonas* (468.3 μg g^{-1}) considerably increased the protein content. Amongst all the inoculation treatments, MAP recorded the highest value of 590.0 μg g^{-1}, which was significant over all other treatments except M_0AP (Table 12). The protein content recorded on 120 DAT showed the same trend as that observed on 60th day. A significant increase in protein content was recorded in all the inoculation treatments over control. Amongst the individual treatments, M_0A_0P recorded the highest value of 701.6 μg g^{-1} followed by M_0AP_0 with 496.6 μg g^{-1} and MA_0P_0 with 490.0 μg g^{-1} as against 351.6 μg g^{-1} noticed in control (Table 12). In the dual inoculations MA_0P showed the maximum value of 675.0 μg g^{-1} followed by MAP_0 with 656.3 μg g^{-1} and M_0AP with 620.3 μg g^{-1} protein content. As it was observed on 60th day, the combination treatment, MAP recorded highest value of 720.0 μg g^{-1}, which was significant over all other treatments except *Pseudomonas* alone treatment. It was noticed that by 120th day AMF showed much influence on protein content of the plants compared to 60th day.

4.2.7 Total Carbohydrate Content (mg g^{-1} leaf tissue) of Tissue Culture Plantlets of Black Pepper as Influenced by Microbial Inoculants

The influence of microbial inoculants on total carbohydrate content of the plantlets was analyzed on 60 and 120 DAT. There was a general

Table 13. Effect of inoculation with different microbial inoculants on total carbohydrate content (mg g^{-1} leaf tissue) of tissue culture plantlets of black pepper

Treatments	60 DAT	120 DAT
MA_0P_0	46.60	50.66
M_0AP_0	43.83	52.30
M_0A_0P	39.10	49.33
$M_0A_0P_0$	38.30	44.30
M_0AP	48. 06	53.60
MA_0P	43.13	53.26
MAP_0	61.43	60.66
MAP	57.86	58.53
CD (0.05)	6.24	5.71

increase in the carbohydrate content in inoculation treatment over control (Table 13). On 60th day of observation, amongst the three organisms, mycorrhiza recorded the highest carbohydrate content of 46.60 mg g^{-1} followed by *Azospirillum* with 43.83 mg g^{-1} and *Pseudomonas* having 39.10 mg g^{-1} leaf tissue. The control treatment recorded total carbohydrate content of 38.30 mg g^{-1} leaf tissue. The increase due to AMF was significant over control and M_0A_0P. In the dual inoculation treatments, MAP_0 recorded the highest value of 61.43 mg g^{-1}, which was significantly higher than all other treatments except MAP. M_0AP and MA_0P recorded carbohydrate content of 48.06 and 43.13 mg g^{-1} respectively. The combination of three organisms recorded total carbohydrate content of 57.86 mg g^{-1} leaf tissue. It was found that AMF and *Azospirillum* considerably increased the carbohydrate content and their combination further augmented the effect. On 120 DAT, among the individual inoculations, M_0AP_0 recorded the highest value of 52.30 mg g^{-1} followed by MA_0P_0 with 50.66 mg g^{-1} which were significantly higher than that of control (44.30 mg g^{-1}). M_0A_0P showed total carbohydrate content of 49.33 mg g^{-1} leaf tissue. All the dual inoculation treatments showed significant increase in carbohydrate content over control. Amongst treatments, MAP_0 recorded highest value of 60.66 mg g^{-1}, which was significantly higher than the values noticed for M_0AP (53.60 mg g^{-1}) and MA_0P (53.26 mg g^{-1}). MAP recorded carbohydrate content of 58.53 mg g^{-1}, which was on par with MAP_0

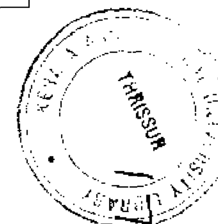
4.3 EFFECT OF MICROBIAL INOCULANTS ON FOOT ROT INCIDENCE AND INTENSITY OF BLACK PEPPER PLANTLETS

The effect of microbial inoculants *viz.*, AMF, *Pseudomonas* and *Azospirillum* on incidence and intensity of foot rot disease in tissue culture plantlets of black pepper was studied and data is presented in Table 14 (Fig. 7). The observations on percentage of plants infected and mortality rate were recorded on 10, 20, 30, 40, 50 and 60 days after inoculation with

Table 14. Effect of inoculation with microbial inoculants on foot rot incidence and intensity of tissue culture plantlets of black pepper

Treatments	Plants infected (number)						Mortality of plants (number)					
	10 DAI	20 DAI	30 DAI	40 DAI	50 DAI	60 DAI	10 DAI	20 DAI	30 DAI	40 DAI	50 DAI	60 DAI
MA_0P_0	3 (25)	4 (33.3)	4 (33.3)	6 (50)	6 (50)	6 (50)	- (0)	3 (25)	4 (33.3)	4 (33.3)	4 (33.3)	4 (33.3)
M_0AP_0	1 (8.3)	6 (50)	8 (66.6)	12 (100)	12 (100)	12 (100)	1 (8.3)	2 (16.6)	7 (58.3)	9 (75)	9 (75)	9 (75)
M_0A_0P	- (0)	2 (16.6)	3 (25)	3 (25)	3 (25)	3 (25)	- (0)	- (0)	1 (8.3)	1 (8.3)	1 (8.3)	1 (8.3)
$M_0A_0P_0$	2 (16.6)	8 (66.6)	12 (100)	12 (100)	12 (100)	12 (100)	- (0)	4 (33.3)	9 (75)	12 (100)	12 (100)	12 (100)
M_0AP	1 (8.3)	3 (25)	3 (25)	5 (41.6)	5 (41.6)	5 (41.6)	- (0)	3 (25)	3 (25)	4 (33.3)	4 (33.3)	4 (33.3)
MA_0P	1 (8.3)	2 (16.6)	2 (16.6)	2 (16.6)	3 (25)	3 (25)	1 (8.3)	1 (8.3)	2 (16.6)	2 (16.6)	2 (16.6)	2 (16.6)
MAP_0	3 (25)	5 (41.6)	5 (41.6)	7 (58.3)	7 (58.3)	7 (58.3)	2 (6.6)	2 (16.6)	2 (16.6)	3 (25)	6(50)	6(50)
MAP	- (0)	3 (25)	3 (25)	5 (41.6)	5 (41.6)	5 (41.6)	- (0)	- (0)	1 (8.3)	1 (8.3)	2 (16.6)	2 (16.6)

Figures in parentheses are values in percentage



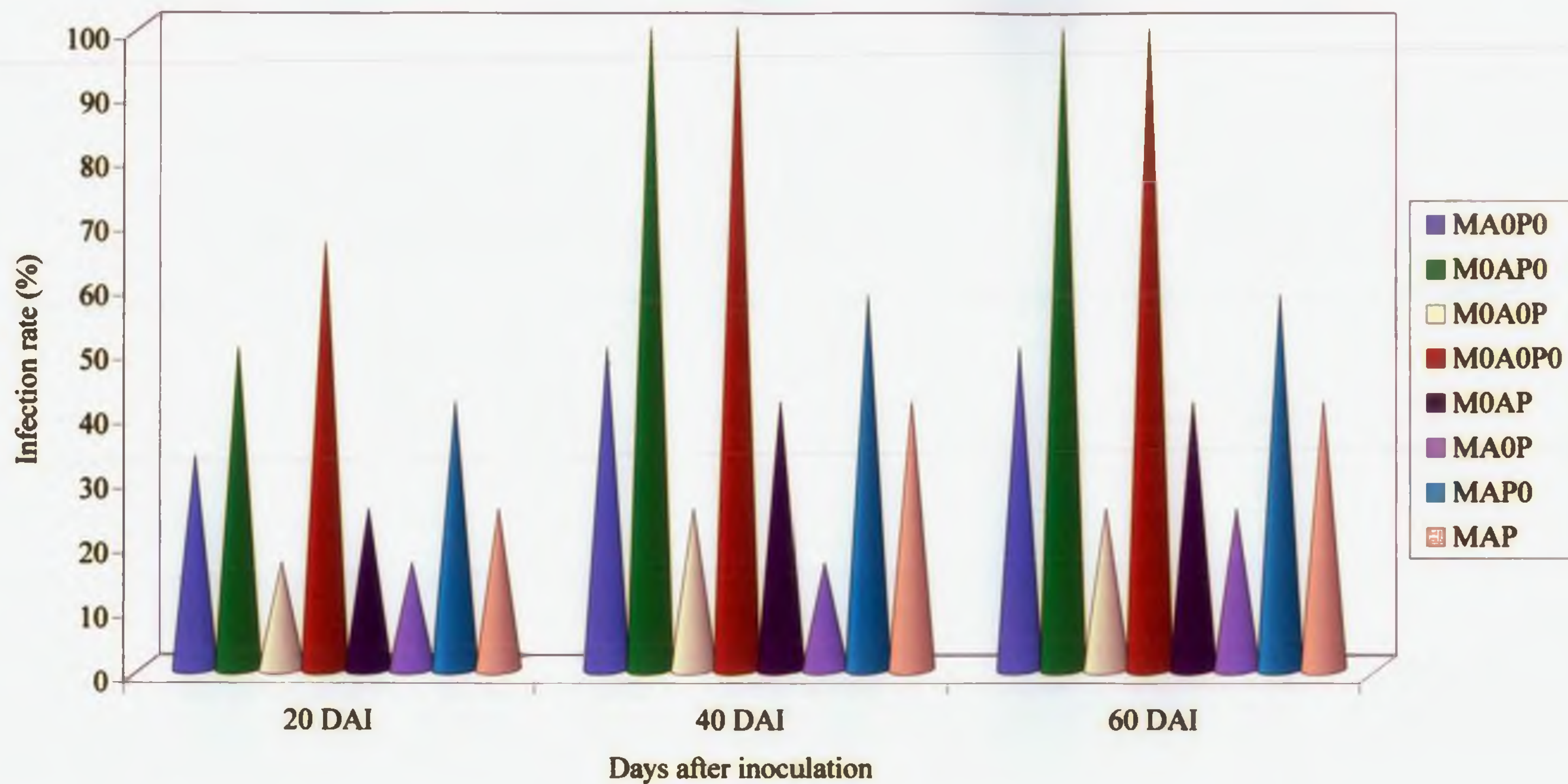


Fig. 7 Effect of inoculation with microbial inoculants on foot rot infection of tissue culture plantlets

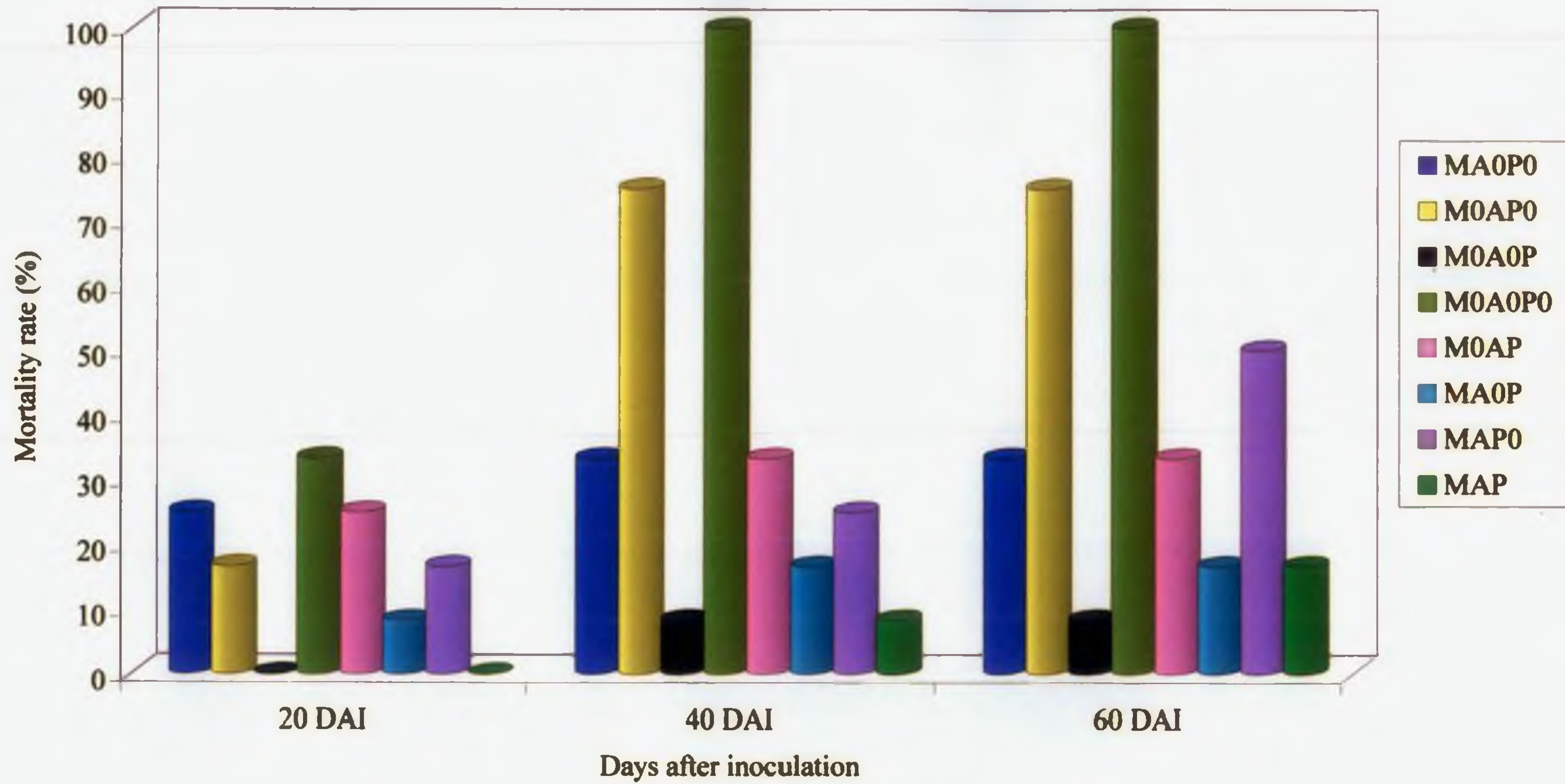


Fig. 8 Effect of inoculation with microbial inoculants on mortality of tissue culture plantlets due to foot rot infection

P - Inoculated with *Pseudomonas*, C - Control

Plate 14 Effect of inoculation with *Pseudomonas* at the time of planting out on foot rot disease of tissue culture plantlets of black pepper

M+P - Inoculated with AMF and *Pseudomonas*, C - Control

Plate 15 Effect of inoculation with mycorrhiza and *Pseudomonas* at the time of planting out on foot rot disease of tissue culture plantlets of black pepper

A+M+P - Inoculated with *Azospirillum*, AMF and *Pseudomonas*, C - Control

Plate 16 Effect of inoculation with *Azospirillum*, mycorrhiza and *Pseudomonas* at the time of planting out on foot rot disease of tissue culture plantlets of black pepper

Phytophthora capsici. On 10th day of inoculation, limited number of plantlets showed *P. capsici* infection. Plants, which received AMF inoculation alone, and the treatment MAP_0 recorded the number of infected plants as three each, whereas treatments M_0AP_0, M_0AP and MA_0P showed infected plant of one each. None of the plants in M_0A_0P and MAP treatments showed infection. Control recorded 2 infected plants (Table 14 and Plate 14). By 20th day, the infection rate further increased. The control recorded 66.6 per cent infection whereas MA_0P_0, M_0AP_0 and M_0A_0P showed 33.3, 50.0 and 16.6 per cent infection respectively. The dual inoculation treatment, MA_0P recorded a low infection rate of 16.6 per cent (Plate 15) followed by M_0AP with 25.0 per cent and MAP_0 with 41.6 per cent. The combination involving all the three organisms (MAP) recorded an infection rate of 25.0 per cent (Plate 16). The 30th day observation showed similar rate of infection in treated plants whereas in control 100.0 per cent infection was noticed. In treatment with *Azospirillum* alone also the infection rate reached 100.0 per cent by 40th day (Plate 17), while other inoculation treatments maintained a low infection percent. The lowest rate of 16.6 per cent infection was recorded in MA_0P followed by treatment with *Pseudomonas* alone with 25.0 per cent. A similar trend with 100.0 per cent infection in M_0AP_0 and control, with relatively low value for inoculation treatments was noticed on 50th day also. The 60th day observation showed a lowest infection percent of 25.0 in *Pseudomonas* alone treatment and MA_0P followed by M_0AP and MAP with 41.60 per cent each. The AMF alone treatment recorded infection rate of 50 per cent (Plate 18) while MAP_0 recorded 58.3 per cent. Amongst the microbial inoculants, *Pseudomonas* or its combination with other microbial inoculants consistently showed relatively low infection rate. Similarly mycorrhiza alone or its combination also showed low infection rate. Treatment with *Azospirillum* did not show much effect on disease incidence when treated alone.

The plant mortality recorded on 10th day of inoculation with pathogen was insignificant (Table 14 and Fig.8). There was notable mortality due to infection from 20th day onwards. No mortality was observed in

A - Inoculated with *Azospirillum*, C - Control

Plate 17 Effect of inoculation with *Azospirillum* at the time of planting out on foot rot disease of tissue culture plantlets of black pepper

M - Inoculated with AMF, C - Control

Plate 18 Effect of inoculation with mycorrhiza at the time of planting out on foot rot disease of tissue culture plantlets of black pepper

A+P - Inoculated with *Azospirillum* and *Pseudomonas*, C - Control

Plate 19 Effect of inoculation with *Azospirillum* and *Pseudomonas* at the time of planting out on foot rot disease of tissue culture plantlets of black pepper

A+M - Inoculated with *Azospirillum* and AMF, C - Control

Plate 20 Effect of inoculation with *Azospirillum* and mycorrhiza at the time of planting out on foot rot disease of tissue culture plantlets of black pepper

Pseudomonas alone and MAP as against 33.3 per cent noticed in control. Individual inoculation with AMF and *Azospirillum* recorded 25.0 and 16.6 per cent respectively. The dual inoculation treatment MA_0P showed 8.3 per cent followed by MAP_0 (16.6 per cent) and M_0AP (25.0 per cent). The mortality rate was further increased by 30th day and the control plants recorded 75.0 per cent mortality, whereas *Pseudomonas* alone and MAP showed 8.3 per cent each. Mycorrhiza alone recorded 33.3 per cent while *Azospirillum* alone recorded 58.3 per cent mortality (Table 14). Amongst the dual inoculations, MA_0P and MAP_0 recorded 16.6 per cent each and M_0AP had 25.0 per cent. By 40th day 100.0 per cent mortality was noticed in control while other treatments maintained a similar trend. On 50th day, the lowest infection percent was in M_0A_0P (8.3) followed by MAP (16.6) against 100.0 per cent in control. Amongst the individual inoculation, M_0AP_0 recorded a higher mortality rate of 75 per cent whereas MA_0P_0 had 33.3 per cent mortality. Amongst the dual inoculations, MA_0P recorded 16.6 per cent mortality followed by M_0AP with 33.3 (Plate 19) and MAP_0 with 50 per cent (Plate 20). The mortality rate recorded on 60th day did not differ from that recorded on 50th day (Table 14). Amongst the 3 microbial inoculants tested, *Pseudomonas* recorded the lowest mortality whereas it was inoculated singly or in combination with others. Similarly AMF alone or in combination also showed relatively low mortality. *Azospirillum* was not that much effective in suppressing foot rot disease compared to *Pseudomonas* and AMF.

4.4 SELECTION OF HOST PLANT FOR *AGROBACTERIUM RHIZOGENES* MEDIATED ROOT TRANSFORMATION FOR AMF PRODUCTION

4.4.1 Selection of host plant

Sensitivity of different host plants such as cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), pigeon pea (*Cajanus cajan*), stylosanthes (*Stylosanthes* sp.) and black pepper (*Piper nigrum*) were

Table 15. Sensitivity of different plant species to infection and transformation for hairy root formation by *Agrobacterium rhizogenes*

Host plant	Time taken for root formation					
	24 hrs		48 hrs		72 hrs	
	Number of roots	Root length (cm)	Number of roots	Root length (cm)	Number of roots	Root length (cm)
Cucumber	2	0.5	5	1.5	8	3
Pigeon pea	-	-	1	0.5	1	1
Tomato	1	0.3	2	0.5	3	0.5
Stylosanthus	-	-	-	-	-	-
Black pepper	-	-	-	-	-	-

tested for *A. rhizogenes* infection and transformation and data presented in Table 15. After inoculation with *A. rhizogenes* the root initiation in the area upto 1 cm above the point of inoculation in the stem was monitored at 24, 48 and 72 hours after inoculation. After 24 hours, 2 roots with 0.5 cm length were noticed in cucumber and in tomato 1 root of 0.3 cm length was observed. Other host plants tested did not show any root initiation in first 24 hours. After 48 hours, there were 5 roots with an average length of 1.5 cm in cucumber along with root initiation in leaves. In tomato, 2 roots with 0.5 cm were noticed. In pigeon pea also one root with 0.5 cm length was observed. Stylosanthes and black pepper did not show any root initiation. After 72 hours, there were 8 roots with 3 cm length in cucumber and tomato recorded 3 roots with 0.5 cm length, while pigeon pea had 1 root with 1 cm length. Stylosanthes and black pepper did not show any root initiation at 72 hours also. Amongst the 3 plants that showed root initiation cucumber showed maximum hairy root formation in the stem. The root initiation from the leaf was noticed only in cucumber. Hence, cucumber was selected for further studies on dual culturing with mycorrhiza (Plate 21).

4.4.2 Inoculation with AMF

After clearing the pathogen, the plants were maintained in rooting medium (Half MS with rooting hormone, IBA) and inoculated with surface sterilized spores of *Glomus monosporum*. The infection and colonization of mycorrhiza could not be achieved since none of the plants survived beyond eighteen days. The attempts made to establish the plant in out door condition in media such as sterilized vermiculite, soil rite, sand, vermiculite – perlite mix, vermiculite – sand mix also failed to achieve establishment of the plant (Plate 22). The separated roots cultured in the rooting medium also did not multiply even after three months. The spores inoculated in such roots in root culture medium though germinated, infection and colonization did not take place.

DISCUSSION

5. DISCUSSION

The beneficial organisms such as AMF, *Azospirillum* and fluorescent pseudomonads are well recognized for their positive influence on nutrition and health of crop plants. There are commendable amount of work in order to exploit these beneficial microorganisms for growth improvement and disease management of crop plants (Okon. 1985; Pacovsky, 1985; Joseph, 1997; Aguilera-Gomez *et al.*, 1999; Sivaprasad *et al.*. 1999b; Pal *et al.*, 2001; Heera, 2002; Baliah *et al.*, 2003). However, the attempts made to exploit these organisms for the benefit of micropropagated plantlets are very meager. Investigation was undertaken to test the effect of inoculation with AMF, *Azospirillum* and fluorescent pseudomonads on survival, growth and incidence and intensity of foot rot disease in micropropagated plantlets of black pepper. Results pertaining to these studies are discussed hereunder.

The inoculation with mycorrhizal fungi and *Pseudomonas* either alone or in combination with other organisms generally improved the survival rate of black pepper plantlets during the hardening phase. Inoculation with *Pseudomonas* resulted in 100 per cent survival of plantlets followed by AMF with 83.3 per cent survival against 66.6 per cent recorded for control. In the present study, though mycorrhiza is well recognized for aiding in the survival and growth of tissue culture plantlets. *Pseudomonas* showed a relatively higher survival rate than mycorrhizal fungi. It is known that the endophytic type *Pseudomonas* brings about series of physiological and biochemical changes in the host plant, which favours the disease resistance and growth of host plant (Burr *et al.*. 1978; Kloepper *et al.*. 1980a; Raaska *et al.*, 1993; Schinder *et al.*. 1994; Defreitas *et al.*. 1997; Paul *et al.*, 2001, 2002, 2003). Further, the growth hormones produced by *Pseudomonas* helps in the early root initiation and development of crop plants (Defreitas and Germida, 1992; Glick, 1995;

Arshad and Frankenberger, 1998; Nowak, 1998; Joseph *et al.*, 2003). *Pseudomonas fluorescens* isolate P-1 used in the present investigation is an endophytic type of bacteria capable of producing higher levels of IAA (Sivaprasad *et al.*, 2003). These desirable traits of the particular organism might have helped in the survival and establishment of plantlets in the hardening phase. The influence of mycorrhiza on survival, establishment and growth of tissue culture plantlets have been well documented in cassava (Calderon *et al.*, 2001), sugarcane (Gosal *et al.*, 2001), strawberry (Taylor and Harrier, 2001; Borrowska, 2002), coffee (Fernandez *et al.*, 2002) and banana (Jaizme-Vega *et al.*, 2002; Lins *et al.*, 2003; Trindade *et al.*, 2003). The high mortality rate of tissue culture plants are attributed to defective physiological features such as poor development of cuticle and epicuticular wax on newly emerging leaves (Lesham, 1983), impaired stomatal mechanism (Capellades *et al.*, 1990), high transpiration rates (Preece and Sutter, 1991) and low photosynthetic rates (Lee *et al.*, 1985). This leads to an uncontrolled water loss from the plant and consequent dehydration and wilting of the plant. Further, the poor development of root and root hair affects the uptake of water and nutrients (Reuther, 1986), and the defective vascular connection between root and shoot hinders the transport of water and nutrients (Sivaprasad and Sulochana, 2004). These defective physiological features seriously affect the water potential of the plant and mortality occurs due to desiccation. Again, since the plants are raised under aseptic conditions and not exposed to any sort of micro flora, the defense mechanism of the plant against the microbial infection is not triggered and hence, they are much vulnerable to microbial infection.

When mycorrhizal colonization takes place, the hyphae ramify inside the root cortical region and spread towards the soil and functions synonymous to root hairs (Miller and Jastrow, 1990; Berta *et al.*, 1990; Schellenbaum *et al.*, 1991). The hyphae take up water and nutrients from the soil and transport to the root cortical region and hence the deficiency

of root hair formation of plantlets is corrected and proper absorption mechanism is provided to the plantlets (Berta *et al.*, 1990; Guillemin *et al.*, 1994; Dhanuja *et al.*, 2002). It could be hypothesized that the mycorrhizal hyphae that ramify in the root cortical region may bridge the vascular connection between roots and shoot, as it is known that the nutrient transport takes place through the hyphae. Hence, there is possibility for correcting the transport of water and nutrients from root to shoot. The physiological changes due to AMF such as increased photosynthetic activity (Sivaprasad and Rai, 1985; Sanchez-Blanco *et al.*, 2004) and enhanced phytohormone activity (Sivaprasad and Rai, 1987) favour the early development of roots and fast growth and development of the plantlets. It is known that AMF induces systemic resistance against pathogens by triggering the production of PR proteins and phenolic compounds (Grandmaison *et al.*, 1993; Sivaprasad *et al.*, 1995a; Gianinazzi-Pearson *et al.*, 1996). This trait of AMF makes the plant tolerant to pathogenic infection also.

Inoculation with *Azospirillum* alone did not show any effect on improving the survival rate of plantlets. Although there was 100 per cent survival in treatment involving *Pseudomonas* alone compared to 83.3 per cent recorded for mycorrhiza alone, it is observed that a loss of 8.4 per cent was noticed during early stages in mycorrhiza treatment. This indicates that the mortality was before establishing mycorrhizal effect on host system as it is known that mycorrhizal infection and subsequent development requires around 15 days. On the other hand, *Pseudomonas* is giving protection to plantlets particularly against pathogenic infection from the time of application. Hence, a combination of *Pseudomonas* and AMF may give better protection to the plantlets. The combination involving either *Pseudomonas* or mycorrhiza also recorded higher survival rate. The combination of *Azospirillum* and *Pseudomonas* and the treatment including mycorrhiza and *Azospirillum* recorded 100 per cent survival each. The combination involving AMF, *Azospirillum* and

Pseudomonas recorded 91.6 per cent survival. Mortality of 8.4 per cent (one plant) occurred immediately after transplanting; perhaps due to the unhealthy condition of the plantlet, as it was evident from the fact that none of the remaining plants showed symptoms of dehydration or pathogenic infection. In the uninoculated control by 30th day of transplanting 33.4 per cent plants died, thereafter there was no mortality. This high mortality during the initial stage was due to wilting consequent to desiccation. In the later stage, the plantlets might have acclimatized with the outdoor conditions and hence there was no further mortality (Fig. 1).

The microbial inoculants have significantly influenced the growth characteristics such as plant height, number of leaves, leaf area and number of branches of tissue culture plantlets of black pepper during hardening phase. The effect on plant height was evident from 30th day after planting. On 90th day after transplanting the effect of microbial inoculants on growth characteristics were more pronounced with maximum plant height for MAP followed by MA_0P. Among the three inoculants, mycorrhiza treatment alone or in combination with either *Azospirillum* or *Pseudomonas* significantly increased the plant height over control. Similarly, *Azospirillum* alone and in combination with mycorrhiza recorded significant increase in plant height over control. Regarding the number of leaves, although, there was no significant difference, the number of leaves was found to increase due to single inoculation or combinations of microbial inoculants over control from 30th day onwards. Similarly, though there was no significant effect on number of branches due to inoculation with different inoculants, there was an increasing trend with microbial inoculant treatments from 30th day after transplanting. *Pseudomonas* was found relatively more stimulatory in branching compared to mycorrhiza and *Azospirillum*.

Inoculation with mycorrhiza either alone or in combination with other two inoculants significantly increased the leaf area of the plantlets from 30th day onwards (Fig. 2). The significant effect of *Azospirillum* and

Pseudomonas on leaf area was evident from 60th day onwards. The observation recorded on plant growth characteristics vividly indicated that inoculation with AMF remarkably increased the growth characters of black pepper plantlets in the hardening phase. Similarly influence of *Pseudomonas* and *Azospirillum* in improving the plant growth characteristics was evident at all stages of observations. It is well documented that inoculation with appropriate mycorrhizal fungus significantly improve the biomass production (Nagarajan *et al.*, 1989; Kumari and Balasubramanian, 1993; Boucher *et al.*, 1999; Mathur and Vyas, 1999; Gardezi *et al.*, 2001) and growth characteristics such as plant height (Shashikara *et al.*, 1999; Gardezi *et al.*, 2001), leaf number (Aguilera-Gomez *et al.*, 1999; Shashikara *et al.*, 1999), leaf area (Aguilera-Gomez *et al.*, 1999; Yano-Melo *et al.*, 1999) and rooting (Anandaraj and Sarma, 1994; Thanuja *et al.*, 2001) of crop plants. The mycorrhiza induced improvement in plant growth characteristics is due to improved nutrient uptake (Harley and Smith, 1983; Joseph, 1997), water relations (Safir *et al.*, 1971, Al-Karaki, 1998; Sanchez-Blanco *et al.*, 2004), photosynthetic activity (Sivaprasad and Rai, 1985; Caravaca *et al.*, 2003; Sanchez-Blanco *et al.*, 2004), phytohormone activity (Allen *et al.*, 1982; Sivaprasad and Rai, 1987) and disease tolerance (Sivaprasad *et al.*, 1995b; Anandaraj *et al.*, 1996; Robert, 1998; Kavitha, 2001; Thanuja and Hegde, 2001).

Azospirillum is a recognized N biofertilizer, which increases plant growth and development through N fixation and hormonal activity (Tien *et al.*, 1979; Kapulnik *et al.*, 1985; Okon, 1985; Hadas and Okon, 1987; Okon and Gonzalez, 1994; Varma, 1995). During the early stages of hardening phase, the hormonal activity might have helped in the root development and growth characteristics of the plantlets and also it contributed in the N nutrition of the plants.

Plant growth promoting rhizobacteria, *Pseudomonas fluorescens* isolate P-1 used in the present investigation is known to produce higher levels of IAA and stimulate the growth and development of black pepper plants (Sivaprasad *et al.*, 2003). This desirable traits of *Pseudomonas* used in the study might have contributed in improving the growth characters of plantlets. The endophytic nature of P_1 may also have further augmented the effect on growth.

The physiological features related to water potential of the plantlets such as stomatal conductance, leaf temperature and relative water content were studied in plants inoculated with different microbial inoculants. There was considerable influence on these parameters at different stages of hardening by the organisms. Stomatal conductance observed on 15 DAT was found relatively low in microbial inoculant treated plants compared to control. On 15th day the stomatal conductance was high in all the treatments compared to 30th and 45th day after transplanting. This may be due to the non-functional nature of stomata at the early stages of plantlets. As the plants acclimatized to the outdoor conditions, the functioning of the stomata gradually reached to normal stage and the conductance was reduced accordingly (Fig. 3). On 15th day, the stomatal conductance recorded for mycorrhiza alone was 0.41 cm s^{-1} as against 0.53 cm s^{-1} recorded for control. *Pseudomonas* inoculation showed 0.41 cm s^{-1} and treatment MA_0P registered the lowest value of 0.33 cm s^{-1}. The relatively low stomatal conductance recorded in mycorrhiza and *Pseudomonas* inoculated plants may be the reflection of the trend in the correction of stomatal functioning taking place; perhaps the better growth and development of the plant due to inoculation favored the stomatal functioning from early stages. On 30th day, the treatment MA_0P_0 recorded 0.18 cm s^{-1} and *Pseudomonas* alone treatment recorded 0.14 cm s^{-1} as against 0.23 cm s^{-1} recorded for control. The treatments MA_0P and MAP recorded 0.15 and 0.12 cm s^{-1} respectively. The relatively lower values recorded for all the treatments is the indication that the functioning of stomata reaching normal.

P. fluorescence isolate P-1 used in the present investigation is known for higher level of IAA production (Sivaprasad *et al.*, 2003).

The data recorded for the relative water content for various microbial inoculant treatments further confirm that the treatments with microbial inoculants increases the water uptake and hence, the water potential of the plant. The relative water content of treated plants was consistently higher at all stages of observation (Fig. 4). On 120 DAT, the maximum RWC being 88.60 per cent recorded for MAP as against 70.40 per cent recorded for control. This amounts to 26 per cent increase in RWC. These observations vividly showed that treatment with microbial inoculants such as AMF, *Pseudomonas* and *Azospirillum* significantly increase the water content of the plant through enhanced uptake from the growing medium, probably due to the improved root development through improved hormonal activity as well as other mechanisms such as better change in kinetics in nutrient uptake as in the case of mycorrhizal system (Berta *et al.*, 1990; Thanuja *et al.*, 2002). This significantly high water content of the plant has definitely helped the plantlets to overcome the excessive water loss and mortality due to the inherent physiological aberrations and registered a higher survival rate.

The total and orthodihydroxy (OD) phenol content of the plantlets treated with different microbial inoculants was found significantly higher at different stages of observation (Fig. 5). The total phenol content was maximum in mycorrhiza inoculated plantlets on 60 DAT followed by different combination treatments involving mycorrhiza. The treatment, MAP recorded 67.33 $\mu g\ g^{-1}$ as against 47.83 $\mu g\ g^{-1}$ observed in control. The increase accounts to 41.00 per cent. A same trend was noticed on 120 DAT also. The OD phenol content recorded in all the treated plants was significantly higher than that observed in control on 60th and 120th day after transplanting. Among the three organisms tested, the effect of *Pseudomonas* was maximum followed by AMF and *Azospirillum*. The

combinations further increased the effect and MAP recorded 44.16 μg g^{-1} as against 16.66 μg g^{-1} recorded in control. A similar trend was noticed on 120th day also. The observations clearly showed that treatment with microbial inoculant significantly increased total and OD phenol content of plantlets compared to control treatment. The plantlets developed in aseptic conditions are not exposed to interaction with any microorganism and hence, the defense mechanism of the plantlets was not triggered (Sivaprasad and Sulochana, 2004). This may be the reason for a very low phenolic content particularly OD phenol noticed in the plantlets at early stages. In the case of plants inoculated with microbial agents got an early opportunity to interact with microorganisms and triggered the defense mechanism of the plant. This led to production and accumulation of phenolic compounds particularly OD phenols. The high values observed in *Pseudomonas* inoculated plants can be attributed to endophytic nature of organism used in the study. The organism invades root, shoot, foliage and other parts of the plants and produces a spectrum of compounds and induces systemic resistance in the plant by the production of defense related compounds including phenolics (Tuzun and Kloepper, 1995; Hallman *et al.*, 1997; Benhamou and Nicole, 1999; Manjula *et al.*, 2002). It is well documented that mycorrhiza invades root cortical region which leads to ISR (Induced Systemic Resistance) in plants as in the case of *Pseudomonas* by the production of defense related compounds including phenolics by the host plant (Grandmaison *et al.*, 1993; Sivaprasad *et al.*, 1995a; Gianinazzi-Pearson *et al.*, 1996). These characteristics of the organism resulted in higher phenolic accumulation in plantlets.

The total soluble protein of the plantlets was significantly influenced by inoculation with different microbial inoculants (Fig. 6). Amongst the three organisms tested, *Pseudomonas* recorded the maximum soluble protein content with 456.0 μg $g^{-1}$and 701.6 μg g^{-1} as against 291.6 and 351.6 μg g^{-1} recorded for control treatment on 60 and 120 DAT

respectively. This was followed by *Azospirillum* and AMF. The combinations of inoculants further augmented the results. The soluble protein pool comprises PR proteins, various enzymes and other protein macromolecules synthesized by the plant. In the present investigation *Pseudomonas* treatment resulted in maximum protein content. This could be attributed to many factors such as better nutrition of the plant, bacteria induced PR protein synthesis and the distribution of bacteria in the plant system. *Pseudomonas* is well recognized for induction of PR protein synthesis in plant system (Kloepper *et al.*, 1980a; Raaska *et al.*, 1993; Defreitas *et al.*, 1997; Paul *et al.*, 2003).

The higher protein level in *Azospirillum* system may be due to the fact that organism fixes N and hence, improves N nutrition and protein content of the plant. Further, the better root development and increased uptake of soil nutrients might have also contributed to high protein content of the plant. Increased uptake of soil nutrients especially K favour the rapid turnover of inorganic N into proteins (Koch and Mangel, 1974). The mycorrhizal association is known to increase the protein content of the plant and suggested at the better nutrition and induction of systemic resistance with PR protein synthesis and other related systems may contribute in increasing the soluble protein (Grandmaison *et al.*, 1993; Gianinazzi-Pearson *et al.*, 1996). The maximum level of soluble protein observed in combination involving all the three organisms may be the reflection of additive effect of the organisms.

Treatment with microbial inoculants generally improved the carbohydrate content of the plants. Maximum carbohydrate content was recorded by AMF and the increase was significant over control, followed by *Azospirillum* and *Pseudomonas*. The combinations of inoculants further increased the effect. It is known that higher carbohydrate content is the reflection of high photosynthetic rate. Mycorrhizal association improves the carbon dioxide fixation rate and increases the carbohydrate content of

the plants (Harley and Smith, 1983; Sivaprasad and Rai, 1985; Rao and Rao, 1998; Davies *et al.*, 2002; Sivaprasad and Sulochana, 2004). Allen *et al.* (1981) attributed mycorrhizal enhancement of photosynthetic rate as a consequence of reduction in stomatal resistance, as observed in the later phase of hardening in the present study, and mesophyll resistance to carbon dioxide uptake and increased chlorophyll content. The better hydration and resultant leaf area increase in plants also favours better photosynthesis (Snellgrove *et al.*, 1982). Relatively higher carbohydrate content observed in *Azospirlllum* and *Pseudomonas* treatments could be attributed to better nutrition and hormonal activity (Okon, 1985; Hadas and Okon, 1987; Raaska *et al.*, 1993; Glick *et al.*, 1994; Arshad and Frankenberger, 1998). This was also evident from the data recorded in the present study that these microsymbionts improve the root development and the growth and development of the plant.

The study conducted to test the effect of inoculation with microbial inoculants, *viz.*, AMF, *Pseudomonas* and *Azospirillum* on foot rot disease incidence and intensity indicated that the association of AMF and *Pseudomonas* remarkably improved the tolerance of plantlets to disease and brought down the incidence and intensity of disease (Fig. 7). On 30th day of inoculation with the pathogen, all the control plants (100.0 per cent) were infected as against 3 (25.0 per cent) and 4 (33.3 per cent) recorded for *Pseudomonas* and AMF respectively.

The mortality of the plants also remarkably reduced due to inoculation with mycorrhiza and *Pseudomonas* either singly or in combination. All the control plants succumb to the disease by 40th day, whereas it was 8.3 per cent in *Pseudomonas* and 33.3 per cent in mycorrhiza treatment. The combination of mycorrhiza, *Pseudomonas* and *Azospirillum* showed 8.3 per cent. These observations vividly indicated the potential ability of AMF and *Pseudomonas* to impart tolerance in tissue culture plantlets of black pepper against foot rot infection and

mortality. *Azospirillum*, though improved the growth and development of the plant, did not show much effect in reducing disease incidence. The mycorrhizal association imparts tolerance against root pathogens and protects the plants against pathogenic infections (Dehne, 1982; Sivaprasad, 1995b; Azcon-Aguilar and Barea, 1996; Cordier *et al.*, 1996; Joseph, 1997; Kavitha, 2001).

Mechanisms of disease control by mycorrhizal association include competition for infection site and host photosynthates (Azcon-Aguilar and Barea, 1996), increased nutrient status of host plant (Dehne, 1982; Hussey and Rancodori, 1982), morphological (Berta *et al.*, 1993) and physiological changes in host root (Snellgrove *et al.*, 1982) – increased ethylene production and DNA methylation by VAM roots (Dugassa *et al.*, 1996), changes in rhizosphere microflora (Azcon-Aguilar and Barea, 1996) and activation of defense mechanisms of host plant leading to hypersensitive response (HR) (Uknes *et al.*, 1996), accumulation of secondary metabolites such as phytoalexins (Harrison and Dixon, 1993) and phenolics (Grandmaison *et al.*, 1993; Sivaprasad *et al.*, 2000) and production of enzymes including PR proteins (Gianinazzi-Pearson *et al.*, 1996).

Similarly, fluorescent pseudomonads are well-recognized biocontrol agents effectively used against most of the fungal and bacterial diseases (Howell and Stipanovic, 1979; Kloepper *et al.*, 1980a; Lifshitz *et al.*, 1987; Laha and Verma, 1998; Rangeshwaran and Prasad, 2000; Heera, 2002; Sivaprasad, 2002; Meenakumari *et al.*, 2003). Major mechanisms involved are siderophore production (Raaska *et al.*, 1993), antibiotic production (Schinder *et al.*, 1994), mobilization of P (Defrietas *et al.*, 1997), inhibition of ethylene synthesis and thus improving plant growth (Glick *et al.*, 1994), and production of growth stimulating phytohormones (Arshad and Frankenberger, 1998). The mycorrhiza and *Pseudomonas* used in the present investigation are known for their potential to suppress

foot rot pathogen, *Phytophthora capsici* (Robert, 1998; Sivaprasad *et al.*, 2000; Sivaprasad *et al.*, 2003).

The tissue culture plantlets developed under aseptic conditions are highly vulnerable to pathogenic infection, as their defense mechanisms have not been triggered earlier. The inoculation with mycorrhiza and *Pseudomonas* stimulate the defense system of the plant at early stage and induces systemic resistance against the pathogens. These factors might have contributed in achieving higher level of protection against foot rot infection and mortality in tissue culture plantlets of black pepper. Hence, the combined application of mycorrhiza and *Pseudomonas* will ensure protection against disease and better growth, development, survival and establishment of black pepper plantlets.

The sensitivity of plant genotypes to *A. rhizogenes* infection and transformation varies. In order to develop AMF production technology in transformed root culture, a sensitive host plant is highly essential. In the present investigation five plant species, *viz.*, cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), pigeon pea (*Cajanus cajan*), stylosanthes (*Stylosanthes* sp.) and black pepper (*Piper nigrum*) were tested for *A. rhizogenes* infection and hairy root formation. Amongst the five plants tested, cucumber showed root initiation above the inoculation point on the stem within 24 hours of exposure to *A. rhizogenes*. The root initiation and elongation was continued in cucumber and within 48 hours five roots with an average length of 1.5 cm was noticed in 1 cm area above the inoculation point. Although pigeon pea and tomato also showed root initiation, it was not as intensive as cucumber. The other two crops did not respond to *A. rhizogenes* inoculation. The root initiation from leaves also noticed in 48 hours in cucumber. The observations vividly showed that cucumber is highly sensitive to *A. rhizogenes* infection and transformation compared to other plants; perhaps cucumber is a sensitive host for the pathogen. The shy root initiation and very slow growth

noticed in tomato and pigeon pea indicate that they are not sensitive hosts for *A. rhizogenes*; perhaps tolerant to the pathogen. *Stylosanthes* and black pepper may be resistant to infection and subsequent transformation. There is no record of work to support this information.

Studies to establish dual culturing with AMF was conducted on transformed roots and whole plant. The separated roots, after clearing the pathogen, did not show root elongation in the root culture medium. The spores placed for colonization in the root, though germinated did not infect and establish colonization in the root. The reasons for such failure of root multiplication and colonization need to be further investigated. The physiological conditions and nutrient concentration of the medium and the root may not favor the infection. Hence, further standardization is required. The attempts made to establish the plant in growing medium also did not succeed. Within 20 days of planting, the plants died due to wilting. This may be due to some physiological changes brought about by *A. rhizogenes* infection such as higher auxins and other hormone concentration in the plant. Hence, in order to achieve successful colonization, further physiological analysis and standardization is required.

SUMMARY

6. SUMMARY

Micropropagation is one of the important contributions of plant tissue culture to commercial plant propagation and has much significance in the rapid production of quality planting material. The major problem that limits the wide spread use of micropropagation is the low survival and growth of plantlets under outdoor conditions. Certain aberrant physiological features of the plantlets often lead to desiccation, wilting and high susceptibility to pathogenic infection.

In the present investigation attempt was made to integrate microbial inoculant technology with micropropagation of black pepper in order to achieve higher survival, growth and foot rot disease tolerance. The microbial inoculants such as AMF, fluorescent pseudomonads and *Azospirillum* were used in the study. Inoculation with different microbial inoculants was given to the tissue culture plantlets of black pepper during planting out. Their survival, growth characteristics and foot rot disease tolerance were monitored. A general improvement in growth and establishment of plantlets was observed due to inoculation with AMF, *Pseudomonas* and *Azospirillum*.

The treatments involving AMF and *Pseudomonas* either alone or in combination consistently showed remarkably higher survival rate compared to other treatments. The combination of mycorrhiza, *Azospirillum* and *Pseudomonas* showed a significant effect on survival and growth characteristics of plantlets such as plant height and leaf area compared to other treatments.

The effect of microbial inoculants on physiological and biochemical characters of the plantlets was also significant. All the treatments involving AMF showed minimum stomatal conductance at early stages of hardening and later gradually increased and recorded a

maximum value of 0.18 cm s^{-1} by combined inoculation of the three microbial inoculants.

In the case of leaf temperature also the treatments involving AMF showed relatively low leaf temperature. The lowest leaf temperature was recorded in combined inoculation of AMF and *Pseudomonas* (32.26°C) on 45 days after transplanting which indicated the higher water content of these plants.

The relative water content of the plantlets was also measured and found that the combined inoculation of AMF, *Azospirillum* and *Pseudomonas* consistently recorded higher values at different stages of observation.

The total phenol content in plant tissues was found to be highly stimulated by AMF (105.83 µg g^{-1}) on 120 days after transplanting followed by *Azospirillum* and *Pseudomonas.* The highest orthodihydroxy phenol content was shown by the treatment MAP (46.66 µg g^{-1}) on 120 days after transplanting. The soluble protein and total carbohydrate content of the plantlet was also significantly enhanced by the combination treatment involving AMF, *Azospirillum* and *Pseudomonas*.

The effect of microbial inoculants such as AMF, *Azospirillum* and *Pseudomonas* on incidence and intensity of foot rot disease in tissue culture plantlets of black pepper was studied. Inoculation with *Phytopththora capsici* was given to incite the disease. Among the three microbial inoculants tested, *Pseudomonas* recorded the lowest mortality whether it was inoculated singly or in combination with others. Similarly AMF alone or in combination also showed relatively low mortality. *Azospirillum* was not effective in suppressing foot rot disease compared to *Pseudomonas* and AMF.

The quality of AMF inoculum is an important factor in tissue culture use. Any technology, which helps to produce AMF in aseptic

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conditions, will be much practical application. Attempt was made to make use of transformed root culture technique using *Agrobacterium rhizogenes*.

In the present study amongst the five host plants tested *viz.*, cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), pigeon pea (*Cajanus cajan*), stylosanthes (*Stylosanthes* sp.) and black pepper (*Piper nigrum*), cucumber was most sensitive for infection and transformation by *A. rhizogenes*. Hairy root formation was observed in 48 hours.

Attempt made to co-culture the transformed root with AMF through root colonization was not successful. The transformed root showed only an insignificant elongation in rooting medium and did not take AMF colonization.

Attempt made to establish the transformed plants in outdoor conditions also failed. Further studies are needed to analyse the physiological reasons for the failure of AMF colonization in the transformed roots so as to further standardize the technique to achieve colonization.

The present investigation vividly showed that inoculation with microbial inoculants – AMF, *Pseudomonas* and *Azospirillum* – is highly beneficial to tissue culture plantlets of black pepper and significant increase in survival rate, growth and tolerance to foot rot disease was achieved. The treatment combination involving all the three organisms was most effective.

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7. REFERENCES

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APPENDIX

APPENDIX – I

Composition of different media

(a) Half Strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962)

Particulars	Weight taken	Volume made up	Volume pipetted
Solution A			
1) NH_4NO_3	16.5 g		
2) KNO_3	19.0 g	250 mg	12.5 ml
3) $MgSO_4 . 7 H_2O$	3.7 g	(10 x)	
4) KH_2PO_4	1.7 g		
Solution B			
1) $CaCl_2 . 2H_2O$	8.00 g	100 ml (20 x)	2.5 ml
Solution C			
1) H_3BO_3	920 mg		
2) $MnSO_4 . 4 H_2O$	1.69 g	100 ml	1 ml
3) $ZnSO_4 . 7 H_2O$	860 mg	(100 x)	
4) KI	83 mg		
5) $Na_2MoO_4 . 2 H_2O$	25 mg		
Solution D			
1) $FeSO_4 . 7H_2O$	745 mg	100 ml	5 ml
2) Na_2EDTA	556 mg	(20 x)	
Solution E			
1) $CoCl_2 . 6 H_2O$	12.5 mg	250 ml	0.5 ml
2) $CuSO_4 . 5H_2O$	12.5 mg	(500 x)	
Solution I			
1) Glycine	200 mg		
2) Nicotine acid	50 mg	100 ml	1 ml
3) Pyridoxine HCl	50 mg	(100 x)	
4) Thiamine HCl	10 mg		

Inositol 100 g
Sucrose 30 g
Agar 8 g

(b) Murashige and Skoog (MS) medium (Murashige and Skoog, 1962)

Particulars	Weight taken	Volume made up	Volume pipetted
Solution A			
5) NH_4NO_3	16.5 g		
6) KNO_3	19.0 g	250 mg	25 ml
7) $MgSO_4 . 7 H_2O$	3.7 g	(10 x)	
8) KH_2PO_4	1.7 g		
Solution B			
2) $CaCl_2 . 2H_2O$	8.00 g	100 ml (20 x)	5 ml
Solution C			
6) H_3BO_3	920 mg		
7) $MnSO_4 . 4 H_2O$	1.69 g	100 ml	1 ml
8) $ZnSO_4 . 7 H_2O$	860 mg	(100 x)	
9) KI	83 mg		
10) $Na_2MoO_4 . 2 H_2O$	25 mg		
Solution D			
3) $FeSO_4 . 7H_2O$	745 mg	100 ml	5 ml
4) Na_2EDTA	556 mg	(20 x)	
Solution E			
3) $CoCl_2 . 6 H_2O$	12.5 mg	250 ml	0.5 ml
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Solution I			
5) Glycine	200 mg		
6) Nicotine acid	50 mg	100 ml	1 ml
7) Pyridoxine HCl	50 mg	(100 x)	
8) Thiamine HCl	10 mg		

Inositol 100 g
Sucrose 30 g
Agar 8 g

(c) Nitrogen Free Bromothymol Blue (NFB) Medium (Baldani and Dobereiner, 1980)

Malic acid	:	5 g
Dipotassium hydrogen phosphate	:	0.5 g
Magnesium sulphate	:	0.2 g
Sodium chloride	:	0.1 g
Calcium chloride	:	0.02 g
Trace element solution	:	2 ml
Alcoholic solution of Bromothymol blue (5%)	:	2 ml
Fe EDTA (1.64% w/v aqueous)	:	4 ml
Vitamin solution	:	4 ml
Potassium hydroxide	:	4 g
Agar	:	1.75 g
Distilled water	:	1 litre
PH	:	6.8

(d) King's B Broth (King *et al.*, 1954)

Peptone	:	20 g
Dihydrogen potassium phosphate	:	1.5 g
Magnesium sulphate	:	1.5 g
Glycerol	:	10 ml
Distilled water	:	1 litre
PH	:	7.2

(e) Yeast Extract Agar (YEA) medium (Ratnasamy, 1997)

Beef extract	:	5 g
Yeast extract	:	1 g
Peptone	:	5 g
Sucrose	:	5 g
Magnesium sulphate	:	300 mg
Agar	:	20 g
Distilled water	:	1 litre

INTEGRATION OF BIOINOCULANT TECHNOLOGY WITH MICROPROPAGATION OF BLACK PEPPER (*Piper nigrum* L.) AND STANDARDIZATION OF PRODUCTION OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF) IN TRANSFORMED ROOTS

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Abstract of the
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ABSTRACT

The present investigation was undertaken to evaluate the effect of inoculation with various microbial inoculants such as arbuscular mycorrhizal fungi (AMF), *Azospirillum* and fluorescent pseudomonads in different combinations on establishment, growth and foot rot tolerance of micropropagated black pepper plantlets and also to standardize transformed root culture technique using *Agrobacterium rhizogenes* for AMF inoculum production in selected host plant.

A general improvement in growth and establishment of the plantlets was observed due to inoculation with AMF, *Pseudomonas* and *Azospirillum*. The combined inoculation of the three microbial inoculants showed a significant effect on survival rate and growth characteristics such as plant height and leaf area. There was significant effect on physiological and biochemical characteristics of the plantlets such as stomatal conductance, leaf temperature, relative water content, total phenol, orthodihydroxy phenol, total soluble protein and total carbohydrate content due to inoculation with microbial inoculants which favoured better survival, growth and disease tolerance of the plantlets. All the treatments involving AMF showed a minimum stomatal conductance at early stages of hardening and later it was steadily increased and reached the maximum. The leaf temperature was found to decrease due to treatments with microbial inoculants. Inoculation with the organisms resulted in consistently high values of relative water content at different stages of observation. Combination involving all the three organisms recorded maximum. The total phenol content in plant tissues was highly stimulated by AMF followed by *Azospirillum* and *Pseudomonas*. The orthodihydroxy phenol, total soluble protein and total carbohydrate content of the plantlets were also significantly improved by inoculation with all the three organisms. Foot rot disease incidence and intensity was

significantly reduced by inoculation with *Pseudomonas* either singly or in combination with others. AMF alone or in combination also showed relatively low mortality due to foot rot disease. *Azospirillum* was not effective in suppressing foot rot disease. In the attempt made to make use of transformed root culture technique using *Agrobacterium rhizogenes* for AMF inoculum production, cucumber was found as the most sensitive host plant. The co-culturing of transformed root with AMF through root colonization was not successful. Further studies are needed to analyse the physiological reasons for the failure of AMF colonization in transformed roots.

The present investigation vividly showed that inoculation with microbial inoculants – AMF, *Pseudomonas* and *Azospirillum* – is highly beneficial to tissue culture plantlets of black pepper and significant increase in survival rate, growth and tolerance to foot rot disease was achieved. The treatment combination involving all the three organisms was most effective.