India is an agricultural country and our economy depends on agricultural outputs. Food grains like cereals, pulses, food additives like spices, condiments, oil seeds like coconut, gingelly, mustard, crop plants like rubber, tea, and coffee etc are the major agricultural crops. Continuous and indiscriminate use of pesticides and chemical fertilizers containing high content of Cd, Zn etc in the field, even though it gives temporary results, but pollute our environment and destroy our micro flora and fauna which in turn affect our agricultural outputs negatively. In such a situation we have to think of alternate measures. Biofertilizers are microorganisms that enrich soil fertility with out causing any harm to the host plants in which they are associated. The unity and interdependence of a healthy plant-soil system is based to a large, but little known and little appreciated, extent on a myriad of microorganisms that inhabit the interface between plant and soil; the rhizosphere. One type of organism stands out among this host of microbes-fungi that penetrate the living cells of plants with out harming them, and whose hyphae at the same time range far in to the bulk soil, establishing equally intimate contact with the micro biota of soil aggregates and micro sites. By doing so these fungi link plant and soil, transporting mineral nutrients to the plant and C compounds to the soil and its biota (Reid,1990). Mycorrhiza; literally means root fungus; have been shown to increase growth and yield of plants. They have been identified with both nutrient mobilization and nutrient cycling. Arbuscular or endo mycorrhizae play a significant role in agriculture and most natural ecosystems, where as ectomycorrhizae have a great potential in forestry and wasteland regeneration. The use of mycorrhizal fungi would reduce dependence on chemical fertilizers besides minimizing environmental pollution (Sharma et al, 1997). They are there fore both agents of plant nutrition and what we will
call Soil nutrition- the VAM fungi (Vesicular Arbuscular Mycorrhiza), tools and technology for use in crop production and soil conservation at the same time. VAM fungi form a fundamental link between the biotic and abiotic portions of the system (O’Neill *et al*, 1991). In all ecosystems, VAM fungi optimize the coupling of plants with rhizosphere microbial processes. These fungi form a symbiotic relationship with plant roots and the fungal symbiont becomes a major interface or connection between the soil and plant. The VAM- symbiosis begins with germination of large spores, or other forms of inoculum propagules, such as root fragments, that occur in most soils as a result of their production and release into the soil from previous host roots. Spores germinate and penetrate the host cortical cells, forming specialized haustoria like structures within the cells called arbuscules, where metabolite exchanges take place between fungus and host cytoplasm. Characteristic vesicles usually also form in the cortical cells and function as nutrient storage organs or as propagules in root fragments. The VAM fungal hyphae also extend from the root, out into the soil where they interface with soil particles. Soil hyphae (extra radical) function as absorptive structures for mineral elements and water. Because they can extend out several centimeters from the root, they can effectively bridge over the zone of nutrient depletion around roots and absorb immobile elements from the bulk soil. Soil hyphae also attract other microbes and together they form water stable aggregates necessary for good soil tilth. The internal colonization of roots by VAM fungi plus the nutrient and water uptake from soil by the soil hyphae of the fungal symbiont causes pervasive physiological changes in the host plant. Such changes make the VAM plant grow and respond to environmental stresses differently from a non-VAM plant.

Cultivation practices like chemical fertilizer and pesticide applications; topsoil removal; long fallow periods; inadequate crop rotation etc will reduce the natural mycorrhizal potential of the soil. That will adversely affect our agricultural outputs. Therefore the incorporation of a
suitable mycorrhizal fungus enables the host plant to overcome these adverse conditions and get positive results. The selection criteria of the best mycorrhizal fungus for a particular host are 1) easy isolation 2) growth rate in pure culture 3) infectiveness 4) effects on host growth 5) ecological adaptation 6) interaction with other microorganisms and 7) host specificity.

Many of the reactions and interactions of the micro flora and fauna that occur in the soil around roots are mediated by VAM fungi that function to deliver mineral nutrients to the host plant in return for a sustained carbon source supply. They also impart other benefits to plants including stimulation of growth regulating substances and alteration of other chemical constituents in the plant; increased rate of photosynthesis; osmotic adjustments under drought stress; enhancement of N\textsubscript{2} fixation by symbiotic or associative N\textsubscript{2} fixing bacteria; increased resistance to pests and tolerance to environmental stresses etc. There is a close relationship between VAM fungi and specific rhizobacteria such as phosphate solubilizers, N\textsubscript{2} fixing bacteria etc. It was suggested that some of the benefits to plant growth attributed to VAM fungi really belong to the combination with their bacterial associates. VA mycorrhizae can play a major role in the quest for sustained plant productivity in all segments of agriculture. It is based on the general hypothesis that agricultural soils have been degraded since the 1800’s from their more microbiologically balanced state of the natural ecosystem before cultivation. Soil degradation involves disturbance of the balance by cultivation, monoculture, excessive inputs of pesticides and chemical fertilizers, removal of organic matter from the soil by crop removal and accelerated decomposition with out replacement and erosion. To maintain economic levels of productivity, farmers have been forced to the practice of indiscriminate usage of chemical fertilizers and pesticides. To overcome these adverse effects, the mycorrhizal fungi and other beneficial microbes need to be established to a high level of effectiveness. According to the food and Agricultural organization of United Nations “Sustainable agriculture
should involve the successful management of resources for agriculture to satisfy changing human needs, while maintaining or enhancing the quality of the environment and conserving natural resources”. It was strongly recommended that the VAM fungi are the universal compensators needed to accomplish the mission of sustainable agriculture.

1.2.1 Objectives of the Study

Keeping all these adverse environmental issues in mind, the study was undertaken to evaluate 1) the presence of type of mycorrhizal fungi in higher plants especially medicinal /economically important plants growing in its natural habitat. 2) Isolation and inoculum production of VAM fungus-
*Glomus mosseae* 3) The tolerance of mycorrhizal plants under various stresses like drought, salinity, heavy metals, pesticide etc- comparison of growth rate, nutrient up take levels, biochemical constituents like total proteins, carbohydrates, chlorophyll, reducing sugars etc; antioxidant enzymes like Super Oxide Dismutase, Catalase, Glutathione S Transferase and antioxidants like Glutathione (reduced), Ascorbic acid etc were studied and compared with that of non-mycorrhizal plants grown under same conditions. 4) Isolation and multiplication of *N₂* fixing bacteria- *Rhizobium leguminosarum* from the root nodules of *Mimosa pudica L* and Phosphate Solubilizing Bacteria (PSB)- *Bacillus* sp from the soil. 5) Co-inoculation of Rhizobium, PSB and VAM fungi and evaluated the level of enzymes like Nitrogenase, Acid Phosphatase and Alkaline phosphatase, responsible for Nitrogen and Phosphorus metabolism, Growth rate and nutrient uptake levels were also estimated and compared. Half of the world’s soils are acid, and crops grown under acidic condition suffer from P deficiency. The soil is acidic because of anaerobic production of CO₂ by microbes. Here comes the importance of mycorrhizae, which can improve P absorption. The tetra partite association of *N₂* fixing bacteria, PSB and VAM fungi could be used as a best biofertilizer in all kinds of soils.
1.2.2 Review of Literature

The term ‘mycorrhizae’ was coined by Frank (1885) for the symbiotic association of fungi with vascular plants. Most vascular plants require mycorrhizae to survive. Glenn, (1982) suggested that families like Brassicaceae and Chenopodiaceae lack functional mycorrhizae because of the presence of glucosinolates and their hydrolysis products; isothiocyanates; in and around their roots. Plenchette et al, (1983) have derived a scheme for calculating relative mycorrhizal dependency index that can be useful in assessing the importance that mycorrhizae have to particular host plants. Mycorrhizal fungi form mutualistic associations with the roots of land plants; providing the plant with phosphorus and other nutrients in exchange for photosynthate. Plants originating from the dry nursery, Nitrogen, Phosphorus, Zinc and Copper concentrations of field grown plants at harvest were significantly increased by pre- inoculation with AMF over those left un- inoculated. The Arbuscular Mycorrhizal Fungal inoculation at the nursery stage under both dry and wet conditions increased growth; grain yield and nutrient acquisition of wet land rice under field conditions. Green house studies were conducted by Audet and Charest (2006) to determine the effect of colonization by the arbuscular mycorrhizal (AM) fungus on the wild tobacco under soil- zinc conditions, reported that the AM plants (particularly roots) showed lower Zn content and concentration than non-AM plants. The AM fungi have a protective role for the host plant, thus playing an important role in soil- contaminant immobilization processes, and there fore are of value in phytoremediation especially when heavy metals approach toxic levels in the soil. Carvalho et al (2006) investigated whether arbuscular mycorrhizal fungi (AMF) increase Cd and Cu uptake and accumulation in the root system of the salt marsh species Aster tripolium L and whether indigenous AMF isolated from polluted salt marshes have higher capacity to resist and alleviate metal stress in A. tripolium than isolates of the same species originated from non-polluted sites. Cd had no effect in root
colonization where as high concentrations of Cu decreased colonization level in plants inoculated with the non-polluted isolate. AM colonization did not increase plant dry weight or P concentration, but influenced root Cd and Cu concentrations. They showed that AMF enhance metal accumulation in the root system of *A. tripolium*, suggesting a contribution of AMF to the sink of metals with in vegetation in the salt marshes. Rabie (2005) had investigated the role of arbuscular mycorrhizal fungal inoculation in red kidney and wheat in heavy metal tolerance in soil artificially contaminated with high concentrations of Zinc, copper, lead and cadmium, found that the metals accumulated by mycorrhizal wheat plants were mostly distributed in root tissues, suggesting that an exclusion strategy for metal tolerance widely exists in them. Mycorrhizal red kidney plants could accumulate relatively high metal concentrations in their shoots, which indicated that internal detoxification metal tolerance mechanisms are also included. AM fungi could be used in plant based strategies of remediation of highly heavy metal contaminated soils. Glass pot experiment was conducted by Zhang, *et al* (2006) to investigate effects of the arbuscular mycorrhizal fungus *Glomus mosseae* on the growth of *Vicia faba* and toxicity induced by heavy metals (Cu, Zn, Pb, Cd) in a field soil contaminated by a mixture of these metals and found that mycorrhizal plants have significantly increased growth and tolerance to toxicity induced by heavy metals compared with non-mycorrhizal plants. Mycorrhizal symbiosis reduced the transportation of heavy metals from root to shoot by immobilizing heavy metals in the mycorrhizal shown by increasing the ratios of heavy metals from root to shoot. Al-Karaki and Clark,(1999) conducted experiments to determine the effects of an AMF and soil ‘P’ on the crop yield, seed protein and lipid contents in two genotypes of durum wheat (*Triticum durum* L). They observed that the percentage of AMF root colonization decreased as ‘P’ added to soil increased and seed protein and lipid were highly correlated with ‘P’ content of plants. VAM association not only augments the nitrogen and
Phosphorus level in leguminous plants but also increases photosynthesis; levels of amino acids, protein fraction and the activity of the enzyme-alkaline phosphatase (Gianinazzi-Pearson, 1978 and Gianinazzi, 1984). The biochemical constituents of root exudates of plants colonized by VAM fungi are reported to have changed and such phenomenon favours root nodulation by *Bradyrhizobium Sp* as well as rhizosphere microbial population (Linderman, 1992). In addition to phosphorus, VAM association also helps in the up take of Zinc and Copper, which ultimately results in the increased plant growth (Gilmore, 1971, Timmer and Leyden, 1978, Bowen, 1980). Arbuscular mycorrhizal fungi are considered as obligate symbionts to crop plants for better utilization of P and other essential elements (Simon et al, 1993). Arbuscular mycorrhizal plants showed increased levels of chlorophyll and carotenoids as compared to non-mycorrhizal plants of *Ziziphus mauritiana* (Paradis et al 1995). Kumutha et al, (2003) reported that the inoculation of arbuscular mycorrhizal isolates increased the leaf biochemical constituents mainly chlorophyll, total carbohydrates & reducing sugars and root phosphate activity in *mulberry cultivar MR2*. VAM fungi depend on plants for carbohydrate and the increased carbon requirement by mycorrhizal plants were compensated by a higher photosynthetic rate as shown in *Citrus aurantium* (Johnson, 1984) and the same kind of result was observed by Pang and Paul (1980) in faba beans.

Higher amounts of P uptake may be the result of higher activity of acid phosphatase as the total and specific activity of this enzyme in root extracts from the inoculated seedlings was found to be significantly higher than the uninoculated control (Choudhury et al, 2002). Kapoor et al, (1988) reported that the VAMF inoculation in the roots of *Trigonella* had increased the activity of acid phosphatase. Straker et al, (1989) suggested that acid phosphatase might play an important role in 'P' nutrition of mycorrhizal host plant.
The roots of AM fungi inoculated plants showed significant increase in acid and alkaline phosphatase activities over uninoculated plants. Acid phosphatase activity of roots was higher, when compared to alkaline phosphatase activity in AM fungi inoculated Papaya (Kennedy and Rangarajan, 2000) in Mulberry (Kumutha et al, 2003) and also in wheat plants (Tarafdar & Marschner, 1994) than their uninoculated control plants respectively.

A major part of the beneficial effects of AM fungi is attributed to their role in ‘P’ uptake and translocation and is reasonable to expect an involvement of phosphatases in the transport of phosphorus. Phosphatase enzymes are directly involved in the intake of phosphorus by plants. Acid phosphatases have been reported in AM fungi and they may be associated with the growth and development of the fungi within the host tissue as well as P acquisition in the rhizosphere (Gianinazzi et al, 1979). High concentration of acid as well as low alkaline phosphatase activity in the rhizosphere of arbuscular mycorrhizal plants which was attributed to direct fungal secretion or an induced secretion of the enzyme by the plants. Acid phosphatase activity increased with the increase in percent mycorrhizal colonization (Sukhada Mohandas, 1992). The phosphates are absorbed by the AM fungi from soil solution as poly phosphate granules localized in the fine branches of arbuscules, which are broken down by specific enzyme activities (Kennady and Rangarajan, 2000). Symbiotic association between microorganisms and higher plants is common and is of great ecological importance in natural and man made biological systems (Garbaye, 1991).

The mycorrhizal association plays an important role in phosphorus cycling and uptake of phosphates by plants. Mycorrhizal plants are therefore adapted to cope with nutrient deficient situations or prevent pathogenesis by other organisms (Fitter and Garbaye, 1994). Leguminous plants can form two types of symbiotic associations with microorganisms. One with *Rhizobium sp* involved in N\textsubscript{2} fixation; the other with VAM fungi...
concerned with the better uptake of P and other nutrients (Crush, 1974). Inoculation of legumes with VA mycorrhizal fungi can stimulate nodulation and N₂ fixation (Mosse, 1981). Dual inoculation with VAM fungi (Glomus mosseae) and Rhizobium showed essential levels of P & N and thus significant increase in biomass productivity in Acacia nilotica (Priya rani et al, 1999) and also in legumes with improved nodulation, N₂ fixation and plant growth (Habish and Khairi, 1970, Hayman, 1978 and 1986). Lakshman and Geeta, (2004) reported that the dual inoculation of VA mycorrhizal fungi, Glomus fasciculatum and nitrogen fixing bacterium, Rhizobium leguminosarum on Acacia nilotica Wild, improved nodulation, VAM colonization, biomass production, nitrogen and phosphorus content when compared to plants which received single inoculation with either organism. Mosse (1988) conducted experiments and concluded that legume inoculation with VA mycorrhizal fungi in phosphate fixing soils was successful, since it not only improved plant growth and nutrition but also enhanced nodulation and crop yield of host plants.

A major beneficial component of soil microbial community is the mycorrhizal fungus; which contributes to plant growth and survival by reducing stresses through symbiosis (Sylvia and Williams, 1992). The interaction between arbuscular mycorrhizae (AM) and minerals other than P; particularly heavy metals, has been the subject of many recent studies because of the possibility of a beneficial effect of mycorrhizae in improving the tolerance of plants against toxicity (Haselwandter et al, 1994).

The effect of an arbuscular mycorrhizal fungus Glomus etunicatum on mineral nutrient, proline and carbohydrate concentrations and growth of soybean (Glycine max) under saline conditions were studied. Salinity decreased AM colonization. When the AM fungus was pre-treated with NaCl with a gradual increase of concentration, and then exposed to a sudden salt stress, their efficiency was increased. This may be due to the acclimation of the AM fungus to salinity (Mozafar et al, 2006).
Chapter 1

The effect of fungicide chlorothalonil on the growth of upland rice, in the absence or presence of the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* were studied by Xu-Hong et al. (2006) and reported that the values of Ascorbate peroxidase (APX), Catalase (CAT) and Peroxidase (POD) activities were reduced more in Mycorrhizal plants than in non-mycorrhizal plants and the results showed that chlorothalonil induced oxidative stress in upland rice and it is needed to evaluate the side effects of chlorothalonil on rice and AMF. Keunho Cho et al. (2006) reported that the arbuscular mycorrhizal symbiosis can confer increased host resistance to drought stress and it also increased the host resistance to salinity stress, in *Sorghum* plants. The production of reactive oxygen species (ROS) is a major damaging factor in plants exposed to different environmental stresses, including salinity (Hernandaz et al., 1995). These oxygen species can seriously disrupt normal metabolism through oxidative damage of lipids and proteins (Jiang and Zhang, 2001). Plants with high concentrations of antioxidants have been reported to have greater resistance to these oxidative damages (Spychalla and Desbough, 1990, Dionisio-Sese and Tobita, 1998, Jiang and Zhang, 2002). Plants have evolved specific protective mechanism, involving antioxidant molecules and enzymes in order to defend themselves against oxidants (Jiang and Zhang, 2002, Nunez et al., 2003). Therefore, antioxidants and antioxidative enzymes such as super oxide dismutase (SOD), Catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APOX) function to interrupt the cascades of uncontrolled oxidants in some organelles, Jiang and Zhang (2001) (Harinasut et al., 2003).

The mycorrhizal colonization on plant growth, nutrient uptake and super oxide dismutase (SOD) and peroxidase (POX) activities in shoots of *Myrtus communis* and *Phillyrea angustifolia* seedlings after well watered, drought and recovery periods were studied by Caravaca et al. (2005) and reported that the peroxidase and SOD activities in inoculated *P. angustifolia* seedlings hardly varied during the drought and recovery periods. SOD activity was enhanced by drought in non-VAM plants of both species and in inoculated *M. communis*, but to a lesser extent than in control plants. The higher enzymatic and non-
enzymatic antioxidant productions would partly alleviate oxidative damage. AM colonization was significantly positively correlated with SOD, APX and $H_2O_2$. AM symbiosis helps in increments of enzymatic and non-enzymatic antioxidant productions, which in turn help AM plants to enhance drought tolerance (Qiang Sheng et al, 2006).

The effect of the arbuscular mycorrhizal fungus, *Glomus versiforme* on growth and reactive oxygen metabolism of trifoliolate orange (*Poncirus trifoliate*) seedlings under well watered (W/W) and water stressed (W/S) conditions were studied by Qiang *et al* (2006, on press) and reported that the increased concentrations of antioxidant enzymes and non-enzymatic antioxidants found in AM plants may survive to protect the organism against oxidative damage, enhancing drought tolerance.

Vesicular – arbuscular mycorrhizae are widely distributed geographically and have been found in association with many economic plants (Gerdemann, 1968). Mycorrhizal infection has particular value for legume because nodulation and symbiotic nitrogen fixation by *rhizobia* require an adequate phosphorus supply and restricted root system of legume leads to poor competition for soil phosphorus (Carling *et al*, 1978). Several legumes grow poorly and fail to nodulate in autoclaved soil unless they were mycorrhizal (Asai, 1944). This is probably due to phosphorus deficiency; since adequate phosphorus is important for satisfactory nodulation and nitrogen fixation (Hayman, 1986). Sitaramaiah *et al*, (1998) reported that the inoculation of *Glomus fasciculatum* on maize plants showed increased vegetative growth; total chlorophyll content and uptake of nutrients viz, nitrogen, phosphorus, potassium, calcium and magnesium over non- mycorrhizal maize plants. The Vesicular arbuscular mycorrhizal (VAM) fungi; a unique group of soil fungi forming symbiotic association with higher plants, facilitate uptake of diffusion-limited plant nutrients such as P, Zn, and Cu (Tinker, 1984). Nitrogen and crude protein content of the neem plants inoculated with *G.mosseae* + *A. chroococcum* was more in shoot
compared to root. The dual inoculation resulted in an increase of 89% in N content and 79% in crude protein content of shoot compared to single inoculated or uninoculated plants (Sumana & Bagyaraj, 2002) and also in maize plants as observed by Barea et al, (1983). Al-Garni (2006) reported that the dual inoculation with arbuscular mycorrhizal (AM) fungus and Rhizobium (N-fixing bacteria) on the host plant cow pea (Vigna sinensis) in pot cultures at different concentration of Zinc (up to 1000mg/kg soil) and Cd (up to 100mg/kg soil) showed significant increase in dry weight, root: shoot ratios, plant length, leaf pigments, total carbohydrates, N and P content than control plants. It provides evidence for benefits of nitrogen fixing bacteria to AM fungi in the protection of host plants against the detrimental effects of heavy metals. If so, bacterial- AM legume tripartite symbiosis could be a new approach to increase the heavy metal tolerance of legume plants under heavy metal polluted soil.

Mycorrhizal fungi form mutualistic associations with the roots of land plants; providing the plant with phosphorus and other nutrients in exchange for photosynthate (Allen, 1991, Smith and Read, 1997). Singh and Kapoor, (1999) reported that the inoculation of wheat plants with phosphate solubilizing microorganisms (PSM) Bacillus circulans and Cladosporium herbarum and the vesicular arbuscular mycorrhizal (VAM) fungus Glomus sp with or without mussoorie rock phosphate amendment showed significant increase in the grain and straw yields as well as N & P uptake. The combined inoculation with PSM and a VAM fungus along with mussoorie rock phosphate amendment can improve crop yields in nutrient-deficient soils. Co-inoculation of Phosphobacteria and Rhizobium with endomycorrhizal fungi, had improved plant growth, nodulation and mycorrhiza formation in Medicago sativa (Aguilar & Barea, 1978). Dual inoculation of soils with mycorrhizal fungi and microorganism Penicillium bilai that can help solubilize rock phosphate; can enhance growth responses of plants in calcareous soils (Kucey, 1987).