PRODUCTION OF ALGINATE ENTRAPPED VA MYCORRHIZAL INOCULUM

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PRODUCTION OF ALGINATE ENTRAPPED VA MYCORRHIZAL INOCULUM

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For my Pappa

With love and remembrances.

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CERTIFICATE

This is to certify that the thesis entitled "**PRODUCTION OF ALGINATE ENTRAPPED VA MYCORRHIZAL INOCULUM**" submitted in partial fulfilment of the requirement for the degree of MASTER OF SCIENCE in AGRICULTURAL MICROBIOLOGY to the University of Agricultural Sciences, GKVK Campus, Bangalore, is a record of research work carried out by **Miss Rashmi Alias** during the period of her study in this university under my guidance and supervision, and this thesis has not previously formed the basis for the award of any other degree, diploma, associateship, fellowship or other similar titles.

Bangalore
September 1995

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INTRODUCTION

I. INTRODUCTION

Mycorrhiza is the unique symbiotic association between roots of higher plants and fungi. Of the different types of mycorrhizal fungi, vesicular-arbuscular mycorrhizal (VAM) fungi are the most predominant type. The VAM fungi is geographically ubiquitous and virtually all vascular families have members with proved VAM associations (Mosse *et al.*, 1981; Harley and Smith, 1983). The ability of the VAM fungi to produce dramatic response in plant growth is well documented (Bagyaraj and Manjunath, 1980; Hayman *et al.*, 1981). The improved plant growth is attributed to increased nutrient uptake especially phosphorus, production of growth promoting substances, tolerance to drought, salinity and transplant shock, resistance to plant pathogen and synergistic interactions with other beneficial soil microorganisms. However, harnessing of these beneficial effects of VAM association for commercial utilization is restricted and minimal. The main reason for this is the difficulty in large scale inoculum production, the fungi being an obligate symbiont and hence cannot be cultured on nutrient or synthetic laboratory media (Jeffries, 1987). This is the major obstacle for popularising VAM fungi for agronomic use.

The obligate symbiotic nature of VAM fungi dictates that this fungal inoculum must be multiplied in association with plant roots in an appropriate medium. The most common method extensively used to raise mycorrhizal inoculum is through pot cultures. Soil containing the spores, mycelium and infected plant roots of VAM fungi is commonly the VAM inoculum used. (Menge, 1983; 1984). Other inert materials have also been tried to mass multiply the VAM inoculum in pot cultures. Hydroponic and aeroponic systems are the other two important methods employed to produce VAM fungal inoculum on a commercial scale.

Formulations of the inoculum before application is essential to concentrate the inoculum and also to facilitate efficient transport, distribution and placement of the inoculum in fields either manually or through currently used agricultural machinery (Fravel *et al.*, 1985; Sylvia and Jarstfer, 1992). Several formulations for VAM inocula are presently available. Important among them are VAM slurries, VAM pellets and encapsulation of VAM in gels.

Immobilization by entrapment in alginate has been successfully used to formulate various microbial inoculants like *Rhizobium* (Hegde and Brahmaprakash, 1992), *Azospirillium* and *Pseudomonas*, (Bashan, 1986), ectomycorrhiza (Cudlin *et al.*, 1992), pesticides, (Pussemier *et al.*, 1992) and potential biocontrol agents (Fravel *et al.*, 1985). Immobilization procedures can preserve the physiological properties of VAM fungi (Strullu and Plenchette, 1990). Strullu and Plenchette (1991) entrapped intraradical forms (vesicles and mycelial fragment) and roots of VAM fungi in calcium alginate beads and used it to inoculate leek plants. The VAM entrapped alginate beads successfully colonized roots of leek plantlets. However, these scientists did not determine the VAM fungal infective propagules number and the shelf life of these propagules in the beads. They have also not investigated the influence of inoculation of these beads on plant growth and nutrition.

The medium which is used to formulate VAM inoculum should maintain the viability and infectivity of VAM fungal propagules, and even after a relatively long period of storage should induce infection of host plants and improve their growth. With this in view it was contemplated to investigate on,

a) The possibility of producing different carrier based alginate entrapped VAM inoculum.

b) To determine the number of infective propagules in the different carrier based alginate entrapped VAM inoculum.

c) To determine the shelf life of the different carrier based VAM alginate beads.

d) To study the response of cowpea to inoculation with different carrier based alginate entrapped VAM inoculum..

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

MYCORRHIZA

Mycorrhiza is the mutualistic symbiosis between soil borne fungi and roots of higher plants. A.B. Frank, a German Botanist coined the term 'Mycorrhiza' in 1885, which literally means 'fungus roots' to describe this unique beneficial mutualistic association.

The two main types of mycorrhizal association are ectomycorrhiza and endomycorrhiza (Harley and Smith, 1983). In ectomycorrhiza the fungus grows intercellularly in the cortex of plant roots to form a distinct network called as 'Hartig net'. The fungus also forms a thick hyphal mantle around the feeder roots, thus altering the morphology of the root. Ectomycorrhizal associations are commonly found in the temperate forest trees. In endomycorrhiza the fungus grows inter and intracellularly and forms specific fungal structures within cortical cells . Arbutoid, Monotropoid, Ericoid, Orchid and Vesicular-arbuscular (VA) mycorrhiza are the different groups which come under endomycorrhiza. It is the vesicular-arbuscular mycorrhizal (VAM) association which are the most important and have the most wide distribution of host plants, while the other endomycorrhizal associations have a limited and specified host range.

VAM FUNGI: TAXONOMY AND OCCURRENCE

A revised classification (Morton and Benny, 1990) places all the soil borne fungi that form arbuscules under a new order Glomales. The order Glomales has two suborders. Suborder Glominae has four genera of arbuscular fungi *Glomus*, *Sclerocystis*, *Entrophospora* and *Acualospora* which form intraradical vesicles. *Gigaspora* and *Scutellispora* are the fungi which form extraradical auxillary cells and no intraradical vesicles are grouped under suborder Gigasporinaeae. The order Glomales belongs to the class Zygomycetes of the sub division Zygomycotina and division Amastigomycotina.

VAM fungi have aseptate mycelium and its infection does not change the morphology of the plant roots. On root colonization the VAM fungi produces two specialized structures known as vesicles and arbuscules in the cortex region of the root (Bowen, 1987). Vesicles are apical or intercalary swellings of hyphae which contain lipids and serve as reserve organs of the fungus. Arbuscules are finely branched hyphae which serve as intensive connection between the fungus and the plant for the transfer of metabolites and nutrients. Attempts to culture VAM fungi apart from their hosts have failed miserably therefore they are considered as obligate symbionts (Jeffries, 1987). VAM fungi are mostly not host specific but experiments have proven that the benefits derived by plants vary with the specific isolates of VAM fungus associated with roots (Bagyaraj *et al.*, 1989).

VAM fungi are almost omnipresent in the natural ecosystem. Virtually all vascular plant families have members with proved VAM association (Harley and Smith, 1983; Trappe, 1987), although it may be rare or absent in families such as Caryophyllaceae, Cruciferae, Chenopodiaceae and Cyperaceae (Hirrel *et al.*, 1978; Bowen, 1987). In addition to the widespread distribution of VAM throughout the plant kingdom this mutualistic symbiotic association is geographically ubiquitous and occurs in plants growing in artic, temperate and tropical regions. VAM fungi occur over a broad ecological range, from aquatic to desert environment (Mosse *et al.*, 1981).

They have been reported to be associated with plants grown in sand dunes, coal mines (Khan, 1978) and aquatic environment (Bagyaraj *et al.*, 1978). However these endophytes do not develop in nitrogen fixing nodules (Lamont, 1972) and anaerobic soil conditions (Tester *et al.*, 1987).

BENEFITS OF VAM ASSOCIATION

Though the phenomenon of mycorrhiza was discovered in the last century itself, it was virtually ignored by most soil and plant scientists and VAM was not considered as a soil microbiological resource for crop production. In the last three decades controlled greenhouse studies have demonstrated that VAM fungi are associated with most crop plants and can improve crop growth and yield by several mechanisms it has attracted the interest of scientists from several disciplines (Powell and Bagyaraj, 1984).

Benefits of VAM association include (Jarstfer and Sylvia, 1993). 1) improved uptake of diffusion limited macro and micro nutrients, 2) increased tolerance to abiotic and biotic stresses and 3) beneficial alterations of plant growth regulators. These benefits result from root interactions which are complex and dynamic. Many depend on the physical, chemical and biological composition of soil.

Enhanced plant growth due to inoculation with VAM fungi was reported by several workers. Legumes (Pairunan *et al.*, 1980; Islam and Ayanaba, 1981), field crops (Black and Tinker, 1977; Lu and Miller, 1989; Baon *et al.*, 1993), forest trees (Saif, 1987; Bagyaraj *et al.*, 1989) and horticultural crops (Vinayak and Bagyaraj, 1990; Michelini and Nemec, 1992) have shown improved growth due to inoculation with VAM fungi.

The principal benefit conferred by the mycorrhizal condition is the increased availability of plant nutrients of low diffusion co-efficient particularly phosphorus. Mycorrhizal plants have a higher amount of P,N,Ca,Cu and Zn than non mycorrhizal plants (Ross and Harpar, 1970; Ross, 1971; Kothari *et al.*, 1991). Soil hyphae of VAM fungi allow the root system to exploit greater volume of soil P by 1) extending away from the root and translocating P from as far as 8 cm (Rhodes and Gerdemann, 1975), 2) exploiting smaller soil pores as VAM fungal hyphae are less than 20 per cent of the diameter of root hairs and 3) adding surface area to the adsorptive system (O'Keefe and Sylvia, 1990). VAM fungi have their most significant effect on plant growth when little phosphate is present in soil (Harley and Smith, 1983).

A mycorrhizal association is known to alleviate water stress in plants (Krishna and Bagyaraj, 1985; Nelsen, 1987), by altering the physiology of the plant. *Sorghum bicolor* and *Eupatorium odoratum* benefitted from VAM fungal colonization when exposed to water stress in two tropical soils (Sieverding, 1981). Drought tolerance of VAM plants was attributed to improved P nutrition in the plant (Nelsen, 1987).

Fungal and nematode plant pathogens interact with VAM fungi in the rhizosphere. Inoculation with the mycorrhizal fungus, *Glomus mosseae* protected the marigold plant (*Tagetus erecta*) against *Pythium ultimum* (Calvet *et al.*, 1993). Occupation of the root cortex by VAM fungi reduced colonization of that zone by the pathogens (Bagyaraj, 1984; Price *et al.*, 1990; Huzhengjia and Guixiangdong, 1991). Therefore, the plants usually benefit from VAM colonization in biotic stress conditions.

Plant growth regulators like cytokinin, gibberellin and abscisic acid in plants have been reported to increase by VAM colonization (Allen *et al.*, 1982; Barea and Azcon-Aguilar, 1982).

In addition, mycorrhizal plants have shown greater tolerance to toxic heavy metals, saline soils, high soil temperature and to transplant shocks than the non mycorrhizal plants. Because of these attributes mycorrhizae are now considered important in the reestablishment of plants in inhospitable sites such as coal and copper mine wastes, burrow pits and badly eroded locations (Hall, 1980).

Thus the ability of the VAM fungi to bring about a dramatic response in plant growth at different soil and climatic conditions is well documented. So there is an increasing recognition in the past thirty years on the role of VAM fungi in the mineral nutrition of many plants, particularly under conditions of suboptimal nutrient supply. Therefore at this juncture, the production of consistently high quality, disease free, clean VAM inoculum on a commercial basis for large scale agricultural use to harness the benefits of VAM symbiosis is pertinent.

HIGH QUALITY VAM INOCULUM PRODUCTION : PREREQUISITES

Certain requirements of VAM inocula are universal whether production is by the most advanced or simplest technique.

Inoculant screening:

Inoculum production will impose additional selection requirement for potential '**inoculant fungi**'. Fungi that are unable to tolerate conditions imposed during or after inoculum production, will be unacceptable as inoculants. Extensive propagule formation and resistance of propagules to desiccation are the two most important factors (Sylvia and Burks, 1988). The VAM fungi, thus screened and multiplied on large scale by a suitable method should have the ability to colonize roots rapidly when inoculated. It should adsorb P and transport it to the host; thus enhancing plant growth. Furthermore the VAM fungi should have the ability to persist in soil and also to reestablish mycorrhiza in the following seasons (Abbott *et al.*, 1992).

Inoculum characteristics:

An inoculum must be infective for the host which it is targeted. An '**infective inoculum**' is one which has the ability to penetrate and spread in roots of the target crop under production (Abbott and Robson, 1981). When the inoculum produces the desired growth on crops, then it can be termed '**effective inoculum**' (Abbott *et al.*, 1992).

The requirement of a concentrated inoculum is a practical one. The ideal additive is small in dose and large in response. '**Concentrated inoculum**' is less costly to store, transport and apply (Jarstfer and Sylvia, 1993). Getting dramatic growth response using very high rates of VAM inocula is impractical (Powell, 1984). The production of concentrated inocula should yield a product that is not bulky and its production cost should be feasible.

Whatever be the method used to produce the inoculum must not spread unwanted organisms such a deleterious rhizobacteria or plant pathogens (Fry, 1982). Practices that prevent or reduce the transfer of potential contaminants should help to assure a '**pathogen free VAM inoculum**' (Jarstfer and Sylvia, 1993).

The viability of the VAM propagules for a longer duration in the inocula produced is of commercial and practical importance. The inocula stored by any method should not aid the multiplication of pathogens and prevent its use later (Siqueria *et al.*, 1985). A prolonged '**shelf life of the**

inocula' is an added advantage. The stored inocula should infect roots and bring about plant response when inoculated.

'**Propagule density**' refers to the concentration of the infective fungal units in the inoculum and this is best predicted by the most probable number (MPN) method (Porter, 1979). Propagules of VAM fungi may contain asexual spores, azygospores, hyphae in dead root fragments, hyphae (+ vesicles) in living roots and extra radical hypha (Tommerup, 1992). These propagules and its density play a crucial role in the rapid colonization of roots (Hung *et al.*, 1990) leading to enhanced growth.

The inoculum used should persist in the soils in the form of different propagules of VAM may be as spores, colonized roots or hyphal network and cause '**persistent colonization**' (Fairchild and Miller, 1988), thereby avoiding inoculation very often.

Inoculation with VAM fungi is clearly necessary in phosphate-deficient soils, that have no or less numbers of indigenous populations or have ineffective strains of these symbiotic fungi (Abbott and Robson, 1985a). Under these conditions the production of high quality, clean VAM inocula (bearing in mind the above mentioned factors) on a commercial scale for large scale agronomic use becomes a necessity.

VAM INOCULUM PRODUCTION : METHODS

The benefits of VAM fungi in agriculture are widely known as is the continued failure to culture these Zygomycetous fungi apart from their hosts. Progress in the development of inoculum of VAM fungi has been severely hampered by the inability to grow these symbionts in axenic culture (Jeffries, 1987).

Soil based VAM inoculum

The production of soil based VAM inocula is relatively easy, besides being a well established and reliable technique. VAM fungi can be mass produced only in the presence of a living roots, and therefore the inoculum production requires a host plant, and optimum conditions provided for the growth and reproduction of the fungi.

Quite a large number of host plants have been tested for VAM inoculum production (Bagyaraj and Manjunath, 1980; Sreenivasa and Bagyaraj, 1988a). The host plant that is used should be highly mycotrophic and well adapted to the climatic and soil conditions of the inoculum production site. Therefore a locally available plant, compatible with all VAM fungi and which grows well on the conditions imposed during inoculum production is selected. The host plant should not harbour root pathogenic fungi which gets transferred to VAM inocula and later to plants inoculated. Sometimes the seeds of host plants in the inocula are contaminants which will be introduced to the inoculated field as weed (Sieverding, 1991; Menge, 1984).

All components of the culture system should be disinfected and the host plant sanitized, before initiation of a pot culture of VAM fungi. This is a critical process, the main objective being to kill existing VAM fungi, pathogenic microorganism and weed seeds, while preserving a portion of the non-pathogenic microbial community (Menge, 1984). Pasteurizing soil by heating to a high temperature (Fry, 1982), using aerating steam or commercial soil pasteurizer (Menge, 1983; Sylvia, 1984), fumigation with methyl bromide or other soil applied biocides, autoclaving (Fry, 1982), use of ionizing radiation (Thompsom, 1990; Jensen, 1983) are some of the methods used to disinfect soil. Host seeds may be disinfested using 10 per cent house-hold bleach (0.5-2.5% NaOCl) for 5-15 minutes, with rinsing in water to remove Cl before use (Tuite, 1969).

Optimum conditions should be provided for the VAM fungi to reproduce and multiply on a large scale in a pot culture. The most effective and efficient inoculation method for starting a pot culture is by placing a layer of VAM fungal inoculum 1-2 cm below the seed (Buwalda *et al.*, 1984). With this method less inoculum is needed than when inoculum is dispersed throughout the container.

Light quality and quantity are very important for successful colonization and spore production. Unshaded greenhouses or high intensity metal halide or sodium vapour lamps for supplemented light provided good results (Menge, 1984). Proper aeration is a must as it has an important role in VAM reproduction. Non saturated and non-stressed water conditions are best for spore production in high and low Phosphorus condition (Nelson and Safir, 1982). Sporulation is positively correlated with temperature from 15-30°C for many VAM fungi. At very high temperature the host is stressed and sporulation may decrease (Menge, 1984).

Soil pH and nutrient deficiencies have to be monitored. Responses for phosphorus and nitrogen fertilization are strain dependent and the relative amount of nitrogen and phosphorus directly affects colonization of VAM fungi (Sylvia and Neal, 1990).

Common contaminating organisms in pot cultures are members of the major groups of soil microorganisms like fungi and bacteria (Secilia and Bagyaraj, 1987; 1988). Pesticides are used to get rid of these contaminants and to control pathogens harbouring roots to produce "clean" inocula. Insecticides and nematicides may act against target organism without adversely affecting propagule numbers of VAM fungi (Sreenivasa and Bagyaraj, 1989). Menge (1983) has advocated the use of selective pesticides in VAM inoculum production.

Though the culture of VAM fungi in disinfected soil has been the most used technique for increasing propagule numbers of VAM (Menge, 1983; 1984) it has its drawbacks. Pot culture technique remains much as an art as a science (Wood, 1985). The more serious problem involves maintenance of high inoculum potential as seasonal fluctuations in inoculum quality is observed, and control of contamination by air-borne pathogens and pests. The soil inoculum also has the disadvantage of being bulky and heavy, hence causes problems with transport and commercial distribution (Sieverding, 1991).

Therefore it is recommended to produce soil inoculum at the site (on farm) where it is required. On farm production of soil inocula of VAM has been described by Sreenivasa and Bagyaraj (1988) and Sieverding (1991). The farmers, however need to be trained efficiently, especially in the use of pesticides and fumigating agents for the production of clean and high quality VAM inocula, so that unwanted contaminants or pathogens are not introduced to the farmers field and the benefits of the VAM association are fully realised and harnessed.

Other substrates used to mass produce VAM inocula

Quite a number of inert, organic and inorganic materials were tried experimentally to mass produce VAM fungi by many scientists.

Calcined montmorillonite (Plenchette *et al.*, 1982), vermiculite, bark, hyphum peat moss and sphagnum peat moss (Biermann and Linderman, 1983a), Perlite + soilrite in 1:1 ratio (Sreenivasa and Bagyaraj, 1988c), expanded clay aggregates, LECA (Dehne and Backhaus, 1986), mixtures of peat, bark, perlite and vermiculite (Graham, 1985), sterilized sand mixed with perlite or sponge (Giovannetti and Avio, 1985), vermiculite, peat, sand dust, perlite (Nemec, 1987), are few examples of the soilless substrates. Culturing VAM fungi in soilless media avoids detrimental organisms in soil without sterilization. These media are light in weight, facilitate better aeration and also holds the desired amount of water.

The inhibitory effect of soilless media on VAM colonization and growth is attributed to the inability of the media to absorb phosphorus from soluble P fertilizer. Therefore rock phosphate a slow release form of phosphorus, which allows for VAM colonization and growth response should be used (Graham and Timmer, 1984).

Maximum inoculum production will result from a vigorously growing plant with high rate of colonization. Hence the substrate used should support good plant growth. Diseases on host crop should be controlled. Uncontaminated spores or other sources of VAM inoculum, clean material of a suitable host, dilute fertilizer solution are other requirements of soilless media used for VAM production (Douds and Schenck, 1990a; Jarstfer and Sylvia, 1993).

The different substrates and host plants used should be standardized before and ideal one can be recommended for mass production of VAM fungi

Expanded Clay aggregates for mass production of VAM fungi

Among the different substrates mentioned above expanded clay as a carrier material for VAM fungi (Dehne and Backhaus, 1986) was used for the successful inoculation of many perennial crops like rubber, papaya, casava, citrus sp., cacao and oil palm (Feldman and Idczak, 1992). The survival of VAM fungi in the expanded clay is very high, storage of the inocula upto 5 years did not affect infectivity, the inocula is light and the product is granular (Dehne and Backhaus, 1986). This can be inoculated to crops in field condition by using a modified pneumatic individual seeding drill through which maize seeds and expanded clay particles containing VAM fungi are simultaneously sown (Baltruschat, 1987).

This type of inoculum however requires 5 months for production. The production methodology and the inorganic clay substrate have been patented. Its production and development has been taken over by the Agricultural Genetics Company and information about progress is more difficult to obtain (Jeffries and Dodd, 1991).

Hydroponic and Aeroponic systems to mass multiply VAM fungi

The nutrient film technique (NFT)is a modified culture system in which the plant roots grow in a shallow layer of flowing nutrient solution which is circulated by pump. The pH, nutrient levels of solution and VAM colonization are monitored. The inoculum produced consisted of root mat of 10-15 mm thick with an extensive viel of fungal mycelium and spores formed outside roots (Warner *et al.*, 1985).

Drying of the roots reduces the infectivity of the inoculum, therefore transport and commercial distribution would be a problem. More over high quantities of infected roots were needed for inocula to be competetive with indigenous fungi (400-800 kg/ha for beans at the rate of 2g infected roots/ seed) report Sierverding and Saif (1984).

An alternate method for producing inocula by NFT is by colonizing plants in peat and subsequently growing them by transferring to the NFT channel which provides more aeration than deep hydroponics (Mosse and Thompson, 1984). The peat particles contain the infected roots, external mycelium and spores of VAM fungi. Contamination of the inoculum during storage by non-mycorrhizal organisms frequently occurred in wet inocula but air drying prior to storage overcame this problem. The air dried inoculum contained 18 propagules per gram of sphagnum peat block (Warner *et al.*, 1985). Air drying reduces the infectivity of the VAM peat blocks. During the production process serious problems may arise regarding contamination of the system by root pathogens. Production of such inocula will be restricted to areas where peat mass is available in plenty (Sieverding, 1991). Dehne and Backhaus (1986) used expanded clay pellets instead of peat for the NFT system. The authors report that the VAM fungi sporulated profusely in the cavities of the pellets.

Aeroponic systems was adapted for growing VAM-fungi by Hung and Sylvia (1988), wherein a fine mist of defined nutrient solution is applied to the roots of host plants. Aeroponic cultures have produced spore concentration as high as 50 per gram of colonized root. After cold storage for seven months at 4°C the root inocula was still infective. However the disadvantages of the hydroponic root culture apply to this VAM root inocula also.

Many publications lack a consistent data on the quality and quantity of the inocula produced by these two systems (Jarstfer and Sylvia, 1993). Production of VAM by the hydroponic and aeroponic systems have been patented.

Axenic cultures of VAM fungi

The Ri plasmid of *Agrobacterium rhizogenes*, genetically transformed roots of *Convolvulus sepium* to produce hairy roots under axenic conditions, to which Mugnier and Mosse (1987) inoculated *Glomus mosseae* spores. The transformed roots were colonized by the VAM fungus. Improved root organ culture techniques by Becard and Piche (1992) made it possible to obtain VAM on transformed as well as non-transformed roots. Hence a complete control on the life cycle of a few species of VAM fungi could be obtained. VAM establishment in the dual culture is followed by production of numerous viable spores and external hyphae. Root organ culture does overcome the disadvantages of pot culture system, such as contamination of plant pathogens and seasonal fluctuations in inoculum quality, but it is technically more difficult to produce than pot culture and expensive too (Wood, 1987).

Spores of VAM fungi germinate on nutrient or water agar (Mosse, 1962; Hepper, 1981). The germinating ability, the pattern of germination and the quantity of mycelium produced are characters that show a high degree of variation (Hepper and Smith, 1976). Hyphal growth from spores was stimulated by CO_2 and root exudates, but continued growth was absent. A molecular signal or a cortical cell environment alone or together induce or depress the genes necessary for biotrophic growth. The unknown factor/factors supplied by living root must be identified and reproduced in a defined media, before axenic cultures will be valuable for mass production of VAM fungi (Becard and Piche, 1989b).

VAM INOCULUM FORMULATIONS

VAM inoculum produced from the above methods are usually formulated to facilitate efficient distribution of the inocula and also concentrate the inocula thereby reducing the bulk (Sylvia and Jarstfer, 1992). Inoculum formulation are required, based on whether the cost of concentrating the raw inoculum is offset by lower transport and spreading costs. Freshly harvested soil inoculum is unsuitable for most forms of machine placement as it rapidly blocks pipes and miller rollers, therefore the inoculum is formulated before use (Powell, 1984).

VAM inocula : sieved or chopped

Hayman *et al.* (1981) concentrated the soil inoculum seven fold by wet sieving through a 100 μm mesh sieve, and applied the inoculum by fluid drilling to soil. Soil or soilless cultures of VAM fungi are processed by sieving, chopping mechanically or by hand prior to use (Warner *et al.*, 1985).

Root from culture pots were reduced in size before use as inocula (Hattingh and Gerdemann, 1975). Root alone as well as vesicles and arbuscules separated from root were used as inocula (Biermann and Lindermann, 1983c). Chopped VAM fungal infested roots produced on host plants grown under NFT can also be used for inoculation, but these root bits lose their viability during drying and storage (Hayman *et al.*, 1981). Roots produced aeroponically were sheared in a food processor which reduced its particle size, but increased its propagule density. The sheared inoculum contained 135, 800 propagules per gram dry weight of sheared root material determined by 10 fold dilution series of MPN method. (Porter, 1979).

Shearing resulted in a viable inoculum of small and uniform size. But the aeroponic roots stored for a longer duration at 4°C or air dried roots were not appropriate for shearing (Sylvia and Jarstfer, 1992).

VAM slurry

Seeds and VAM fungal inocula were incorporated into an uniform suspension in a viscous fluid of methyl cellulose and applied to the furrows as a slurry. Though this method proved successful under field conditions, this type of inocula may damage the emerging radicle therefore germination of seeds must be rigorously controlled (Hayman *et al.*, 1981). To the slurry of carboxyl methyl cellulose containing spores and infected roots of VAM fungi, roots of pregerminated plants were dipped before transplanting them (Menge and Timmer, 1982). Slurry prepared by mixing two parts of VAM fungal (*Glomus microcarpum*) spores suspension in one part of steam sterilized lignite was used to inoculate tubers of *Coleus parviflorus*, by soaking the tubers in the slurry for 3 hours. Even after six months of storage the slurry resulted in nearly 100 per cent infection (Potty, 1989). The amount of slurry used to inoculate the tubers has not been mentioned in the paper. The potential for survival of infective propagules of VAM fungi in aqueous slurries is unknown (Sieverding, 1991).

VAM pellets

Seeds were coated with an adhesive (methyl cellulose) to which the culture inocula is expected to stick and subsequently colonize the radicle. Because of the large size of soil particles containing VAM propagules it is difficult to stick to the seed. This method has proved satisfactory for large seeded crops (Hatting and Gerdemann, 1975). Seeds were mixed with VAM inoculum substrates and *Rhizobium* strains before pelleting to obtain multiseeded pellets (Hayman *et al.*, 1981). Only small seeds can be used here and the survival of seeds can be used here and the survival of seeds and *Rhizobium* in these pellets is not known (Sieverding, 1991).

Hall (1979) prepared soil pellets of VAM by mixing 20 parts of sedimentary losse clay, one part of tertiary sedimentary clay and enough water. These pellets contained 20 VAM spores per gram of pellet. Another method to prepare VAM pellets is by using gum arabica (Hall and Nelson, 1981). Pelleting could be a useful method to introduce VAM fungi to sterile soils, however the inoculum potential of the pellets to infect under field conditions requires further research (Hall, 1979). Peat blocks of the hydroponic system were processed and extruded to form pellets which contained the roots, spores and hyphae of VAM fungi and reportedly retained their infectivity upto 6 months of storage (Warner *et al.*, 1985).

Propagules produced on Leca clay were dried prior to use making the VAM inocula granular, thus easy to apply to field (Dehne and Backhaus, 1986).

Hoeflich and Glante (1991) used VAM inocula produced in peat clay substrate with maize host plant, incrusted it to seeds or the inocula granules were drilled together with seeds. By seed or soil application the yield of maize and lucerne increased respectively.

Encapsulation of VAM fungi in gels

Hung *et al.* (1991) tested hydroxyethyl cellulose (Natrosol) as a carrier for VAM fungal inoculum and as a sticking agent for aeroponic production of VAM fungi. They observed that Natrosol is a good carrier for the VAM infested roots. Natrosol had no effect on spore germination and root colonization.

Aeroponically produced sheared root VAM inocula was encapsulated in carageenan and hydroxyethyl cellulose (Natrosol) a gel carrier. The propagule numbers were 1.2 per gram and 0.5 per ml respectively. Increasing, inoculum density resulted in increasing colonization (Sylvia and Jarstfer, 1992).

ALGINATE ENTRAPPED CELLS : USE IN AGRICULTURE

Immobilization of microbial cells is a well known method having potential application in industries for their stability and easy recovery. But in agriculture the immobilized system used should facilitate the release of microbial inoculant to elicit the benefit of plant microbe interactions. Cell immobilization is defined as the attachment in a distinct solid phase that allows exchange of substances, products, inhibitors etc. with but at the same time separates the cells from the bulk phase in which substrates etc. are dispersed and monitored (Tramper, 1990). Entrapment in polymeric networks is a method of immobilization. A subgroup of this is ionotrophic gelation. This procedure is a reversible one therefore recovery of entrapped cells is easy and cell number can be easily determined. Immobilized cells retain high catabolic activity, are protected from predators, parasites and inhibitors and efficiently mineralize substrates (Brodelius and Vandamme, 1987).

Aqueous solutions of sodium alginate and other univalent cation salts of algenic acid form a gel when in contact with calcium or several other divalent cations. This property has been used to prepare beads containing the microbial cells.

Debongie and Pussemier (1992) observed that carbofuran and aldicarb entrapped in alginate beads percolated slowly, at a constant rate taking few hours (aldicarb beads) to 1-5 days (Carbofuron beads) to spread, compared to few minutes of commercial formulation of the same chemical in a soil column. Pussemier *et al.* (1992) compared the effect of dried aldicarb alginate beads and the commercial formulation, Temik 106 on sugar beet. The alginate formulation delayed the release and migration of the active ingredient in soil, reduced the leaching under 8 cm depth, and had only a small effect on the uptake of active metabolites by plants.

Sodium alginate formulation of isolate C20 of *Gleocladium virens* selected to control *Pythium ultimum* and *Rhizoctinia solani* maintained a high population density in dry beads when stored at 4 and 10°C but not at 30°C for 2 months. Alginate beads of *G. virens* when added to soilless mix before planting showed promise in the control of damping off in greenhouse conditions. The overall plant stand of seedlings exposed to *R. solani* inoculum was improved, post emergence damping off was controlled substantially by the alginate formulation of this biocontrol agent (Lumsden and Locke, 1989).

Two soil microalgae *Chlorella vulgaris* and *Scenedesmus bijugatus* entrapped in calcium alginate beads stocked with 108 cells each removed 71-79 per cent more nitrogen and 52-82 per cent more phosphorus within 6 hours compared to those with stocking density of 105 cells per bead (Megharaj *et al.*, 1992).

Samal and Kannaiyan (1992) investigated the effect of inoculation of immobilized strain of *Anabaena azollae*, *Anabaena* sp. and *Nostoc* sp. at the rate of 60 beads (45 algalcells/bead) each on rice cv. IR64. Plant growth was highest with inoculation of *Nostoc* alginate beads as excretion of ammonia was greatest from these beads.

Similarly the immobilization technique can be used for the development of artificial seeds and for co-inoculating these artificial seeds with a microbial inoculant which enhances its germination. Hot water extract from marine cyanobacterium *Synechococcus* sp. and carrot somatic embryos (*Dacus carota*) were encapsulated in calcium alginate gel. The germination percentage in coimmobilization was 91 per cent compared to 35 per cent without adding the extract of *Synechococcus* sp. (Wake *et al.*, 1992).

Romaine and Schlagnhaufer (1992) produced button mushroom (*Agaricus bisporus*) of typical morphology using mycelium - colonized alginate pellets on compost. This technology of using alginate entrapped mycelium as inoculant shows greatest potential as a pathogen free inoculant for commercial production of mushrooms.

Propagule suspension of potential biocontrol agents and pyrax were entrapped in calcium alginate beads. The propagules were viable upto 12 weeks at room temperature in the dry alginate beads. The beads are biodegradable, relatively uniform in size and composition. This formulation facilitates the use of these biocontrol agents with currently used agricultural equipment (Fravel *etal.*, 1985). Alginate films were used to deliver nematode eggs to soil and evaluate the microbial interaction. The number of immature eggs, eggs with first stage juveniles declined linearly over time while the empty egg shells and hatched juveniles increased over time indicating that the alginate gels did not inhibit development and motility of *Meloidogyne incognita* juveniles (Kabana *et al.*, 1994).

Rhizobium entrapped in alginate gel (AER) or a mixture of xanthan and carbgum (XER) were used for soil inoculation. There was no marked differences among the semidried XER, dried XER, dried AER and peat based *Rhizobium* inoculum. Since semi dried XER could be prepared in a granular form and applied readily to soil using common machinery, it was an appropriate form for *Rhizobium* inoculation to soil. The complication of damping off sometimes associated with the peat based inoculants at the time of seeding may be avoided by using XER inoculant (Jung *et al.*, 1982).

Hegde and Brahmaprakash (1992), prepared dry granular inoculant of *Rhizobium* using sodium alginate and perlite. The inoculation of this was comparable to that of peat based inoculant of *Rhizobium*. The alginate granules were free from contamination, could be stored in dry state without losing much viability and could possibly overcome the need for reinoculation in the tropics.

Bashan (1986) used alginate and skim milk for the preparation of *Azospirillium* inoculant. These granules were uniform in composition, supported large population of the inoculant and allowed for slow release of the inoculant over a longer period. Alginate beads were biodegradable and produced no environmental pollution.

Alginate entrapped *Nitrosomonas europea* was used as a biocatalyst for the reduction of NH_4^+ to NO_2. The immobilized cells stored at 4°C were more stable than free cells. The activity of immobilized cells increased considerably because of the multiplication of the cells in the support. Therefore, the operational stability of immobilized *Nitrosomonas europea* showed potential for nitrification of waste waters (Ginkel *et al.*, 1983).

Alginate entrapped ectomycorrhizal inoculum

Ectomycorrhizal inoculum is produced on a large scale by immobilizing the homogenized fungal mycelium along with perlite in sodium alginate. The alginate inoculum was easy for production, application and had a longer shelf life. So it can be used for inoculating forest nurseries, seedling establishment at polluted sites and difficult reforestation areas (Kropacek *et al.*, 1989; Cudlin *et al.*, 1992,). Homogenization of the fungal mycelium resulted in poor physiological status of the fungus and lowered the viability of beads. Hence Mortier *et al.*, (1989) immobilized fungal mycelium grown in vermiculite peat mix in alginate gel for inoculation purposes.

Mycobead was produced by entrapping submerged aerobic culture of ectomycorrhizae mycelia within hydro gel. The hyphae in the beads were whole and intact in contrast to the case where homogenised mycelium was used. Storage of beads upto 7 months did not significantly effect the viability of the Mycobead. The ability of mycelia to grow out of beads indicated high degree of viability. 100 per cent initiation of mycorrhiza was achieved with small doses of Mycobead which is an advantage in production, storage, transport and usage (Kuek *et al.*, 1992).

Dual inoculation with ectomycorrhizal fungus *Laccaria laccata* and together or not with one of the five mycorrhization helper bacteria entrapped in calcium alginate, was suitable for inoculation of commercial forest nurseries and was more efficient than the classical peat vermiculite ectomycorrhizal inoculum (Duponnois and Garbaye 1991).

Alginate entrapped VAM inoculum

With the advantages of using algenate entrapped microbes over the classical method it seems apt to produce a immobilized VAM fungal inocula for inoculation of crop plants.

Strullu and Plenchette (1991) isolated intraradical forms (vesicles and mycelium fragments) of *Glomus* sp. and immobilized them and the roots of VAM fungi by entrapping in alginate beads. Inoculation of these beads resulted in the formation of mycorrhizas in leek plants even after storage at 4°C for one month. The intraradical forms regenerated to produce hyphae thereby colonizing roots. Strullu *et al.*, (1989) suggested that calcium may be important for the regeneration of intraradical forms.

Strullu and Plenchette (1991) have determined the percent mycorrhizal colonization caused by the alginate beads on the day of preparation and one month after storage at 4°C. They suggest that encapsulation in sodium alginate stabilized the physiological properties of mycorrhizal roots and vesicles. However, they have not determined the VAM infective propagule number in these beads. The influence of the alginate beads on plant biomass and phosphorus content of plants was also not studied.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

The present study was taken up to investigate the possibility of producing alginate entrapped VA mycorrhizal inoculum. The beaded VAM inoculum got from amending the sand : soil inoculum with different carrier materials, and by entrapping it in alginate gel was screened for infectivity and viability of VAM propagules and their effect on growth of cowpea.

VA mycorrhizal inoculum

Glomus mosseae (Nicol. and Gerd.) Gerdemann and Trappe maintained as pot culture in sterilized 1:1 (v/v) sand : soil mixture on Rhodes grass (*Chloris gayana* kunth.) in the mycorrhizal culture collection of the Department of Agricultural Microbiology, Gandhi Krishi Vignana Kendra, University of Agricultural Sciences, Bangalore was used in this study. The air dried inoculum contained VAM hyphae, spores and dried root pieces. The number of infective propagules in this inoculum was determined by following the most probable number (MPN) method, using four fold dilutions (Sieverding, 1991).

Production of alginate entrapped VA mycorrhizal inoculum

The air dried sand : soil inoculum of *Glomus mosseae* was passed through 425 μm sieve. Perlite, soilrite, talc vermiculite, kaolinite and bentonite were the different carriers used. Soilrite and vermiculite were ground in a grinder and screened through 425 μm sieve prior to use.

Two per cent sodium alginate (Rolex) solution was prepared in sterile distilled water by heating it to 60°C and mixing it throughly on a magnetic stirrer. Later this solution was cooled to room temperature. To this aqueous suspension 2 per cent of the carrier material and 10 per cent of the sieved sand : soil inoculum was added. The concoction was throughly mixed to get a homogenized mixture. Using a sieve this mixture was added to sterile 0.1 M calcium chloride solution. This process entrapped the sand : soil inoculum of VAM mixed with the carrier material to form calcium alginate beads instantly. The beads were allowed to stay in $CaCl_2$ solution for 30 minutes to harden, and the excess $CaCl_2$ solution was decanted. The beads were washed in running tap water. A part of the alginate entrapped inocula was dried to surface dryness and stored in a conical flask at 5°C in a refrigerator (Kropacek *et al.*, 1989; Strullu and Plenchette, 1991). This formed the wet alginate entrapped VA mycorrhizal inoculum. Another part of the beads was air dried and stored in plastic covers at room temperature. This fraction formed the dry alignate entrapped VA mycorrhizal inocula.

Moisture content of the alginate entrapped VA Mycorrhizal beads

The moisture content of the wet alginate entrapped VAM beads was computed using the weights of wet and dry beads

$$\text{Moisture percentage} = \frac{\text{Weight of wet beads - Weight of dry beads}}{\text{Weight of wet beads}} \times 100$$

Volume of carrier based dry VA mycorrhizal alginate beads

The volume (ml) of the different carrier based dry VAM alginate beads was measured using a measuring cylinder. Five grams of the different carrier based dry VAM alginate beads were transferred to a measuring cylinder, and gently tapped on a table and the volume (ml) was noted.

pH of the different carriers

The pH of the various carriers was determined using a digital pH meter (model 335 systronics). The carrier to water ratio used was 1:10 (w/v).

Inoculum potential of the alginate entrapped VA mycorrhizal inoculum

Infectivity of the alginate entrapped VAM beads was determined by the most probable method (MPN) using four fold dilutions (Sieverding, 1991). This method quantifies all infective VAM propagules and estimates the VA mycorrhizal population giving number of infective propagules (I.P) per unit weight; additionally the limits of confidence of the enumeration can also be calculated.

To 100 g of the wet perlite based VAM alginate beads in a mortar 25 ml of 0.2 M sodium citrate solution (pH adjusted to 7.2) was added. Using a pestle the beads were gently crushed, before transferring them to test tubes. The beads were dissolved by vortexing them. The dissolved beads were mixed with 300 g of carrier (sand : soil mix in ratio of 1:1 (v/v), sterilised by autoclaving at 121°C and 1.1 kg/cm^2 pressure for 1 hour was used after 15 days) throughly to obtain the dilution 4^{-1}.This was dried for a day and four fold dilution series was prepared upto 4^{-10}. The diluted cultures from 4^{-1} to 4^{-10} dilutions were filled into pine cell tubes at the rate of 60g per tube. Five replications per dilution was maintained. Onion seeds (Nasik red variety) surface sterilized using 70 per cent alcohol for 1 minute were sown in the pine cell tubes at the rate of 10-12 seeds per tube. After seedling emergence 5 plants per tube was maintained. Onion was used as test plant as it is highly mycorrhizal and its fine roots facilitate easy staining and observation of vesicles. Onions seedling were grown for 8 weeks in a glasshouse. After 8 weeks of plant growth the roots were harvested and washed to remove adhering soil particles and stained with 0.05 per cent trypan blue in lactoglycerol (Phillips and Hayman 1970). The stained root system was observed using a stereozoom microscope model SZ 1145 TR (Olympus Japan) at 100x magnification.

The presence or absence of VA mycorrhizal colonization was scored as positive (+) or negative (-) in all the replications in each dilution. The most probable number (MPN) of VAM infective propagules (I.P.) was then calculated using the formula.

$$\log \Omega = x.\log a - K$$

where Ω is the number of I.P. of VAM.

x is the mean number of tubes positive for infection.

$$x = \frac{\text{Total number of infected tubes}}{\text{Number of replications per dilution}}$$

a is the factor of dilution which is 4 for four fold dilution K is found in Table VIII of Fischer and Yates (1970) at the determined value of x.

The confidence limits (Ω_s superior, Ω_i -inferior at 95% confidence limits) was calculated using the formula given below.

$$\log \Omega_{si} = \log \Omega \pm \frac{S}{\sqrt{n}} . Z$$

where $S = \sqrt{0.201}$ for four fold dilution.

n = number of replications per dilution.

z = 1.645 for the 95 per cent probability.

The I.P. number was determined in a similar way for the other carrier based alginate beads. For determining the I.P. number of *Glomus mosseae* in sand: soil inoculum the dissolution step was skipped, the rest of the procedure remained same.

The I.P. number was determined for wet beads only. The I.P. number for the dry VAM alginate beads was derived from the value of wet heads by knowing the moisture percentage.

Viability of the alginate entrapped VA mycorrhizal heads

Samples of wet beads stored in conical flask at 5° C and dry beads stored in polythene covers at room temperature (30°C ± 5°C) were taken at 30 day interval upto 180 days to test the viability of the VAM fungal propagules in the different carrier based propagules in the different alginate beads. Five grams of wet and dry alginate beads made using different carriers were dissolved by adding 2.5 ml and 4 ml of 0.2 M sodium citrate solution (pH adjusted to 7.2) respectively and shaken vigorously on a vortex mixer. Three replicates were maintained for each treatment. The solubilized inoculum was mixed with 60 g of sterile sand : soil mix and transferred to pine cell tubes. The tubes were seeded with onion (Nasik red variety) and the seedlings were grown for 8 weeks. The roots were harvested washed clean of soil debris and stained with 0.05 per cent trypan blue in lactoglycerol. The stained roots were observed under stereozoom microscope and per cent root colonization was calculated following the grid line intersect method (Giovannetti and Mosse, 1980).

Response of cowpea to inoculation with alginate entrapped VAM inoculum

Soil

Soil for this green house study was collected from an uncultivated field in G.K.V.K. campus. Soil sample was crushed to pass through 4mm sieve and mixed to obtain a homogeneous mixture. The pH of the soil was determined using a digital pH meter model 335 (Systronics) and was found to be 5.5 (soil to water ratio 1:2.5). The available P per g was 2.4mg (NH_4F + HCl extractable) The mycorrhizal I.P. number in the soil was estimated by the MPN method using four fold dilutions (Sieverding, 1991) and was found to be 0.31 I.P. per gram of soil.

Mycorrhizal inoculation

The pots were filled with 3.5 kgs of sieved soil and mixed well with the required amount of inocula, so that each pot received 500 I.P. of VAM as given below. The different carrier based alginate entrapped VAM inocula were prepared as detailed earlier. Out of the 6 carrier based alginate beaded inocula studied in earlier experiment. Only 4 beaded inocula which supported high propagula numbers of VAM fungi were used in the present study. They were the alginate entrapped wet and dry VAM beads prepared using perlite, soilrite, talc and vermiculite as carrier materials. This experiment consisted of the following 10 treatments.

Treatment number	Carrier	dry/wet	Amount added (g) to each pot to get 500 I.P. of VAM fungi
1	Perlite	wet	131.93
2	Soilrite	wet	174.22
3	Talc	wet	230.41
4	Vermiculite	wet	400.00
5	Perlite	dry	24.65
6	Soilrite	dry	29.34
7	Talc	dry	39.40
8	Vermiculite	dry	65.53
9	Sand: Soil inoculum	dry	10.87
10	Uninoculated control	-	-

Each treatment was replicated 5 times, thus this experimental set up had 50 units.

Seed treatment and Planting

Cowpea (*Vigna unguiculata* (L.) walp.) of variety TVx944-02E was used as host plant to study its growth response to inoculation with alginate entrapped VA mycorrhizal inocula. Cowpea seeds were surface sterilized with 70 per cent ethyl alcohol for 1 minute followed by washing with sterilized distilled water three times. Seeds were later treated with 0.1 per cent $HgCl_2$ for 3 minutes and washed with sterlized distilled water six times. Surface sterilized seeds were pregerminated on 0.9 percent water agar. Five pregerminated seeds were planted per plot. After a week of planting the number of seedlings were thinned to 2 per pot. The plants were harvested after 75 days of growth.

Experimental layout and plant nutrition

The pots were arranged in a glass house in randomized complete block design. Randomisation among treatments was done once in every 20 days. The plants were grown under natural light and were fed with 50 ml Ruakara nutrient solution (Appendix I) thrice during the 75 days period of growth. The initial application was complete nutrient solution with phosphorus. The later two applications were with nutrient solution minus phosphorus.

Observations recorded

Plant height :

Plant height was recorded from the collar region to the base of the fully opened leaf at the tip of the plant on the 20th, 40th, 60th and 75th day after planting.

Number of trifoliate leaves

The number of fully opened trifoliate leaves were counted on the 20th, 40th, 60th and 75th day after planting.

VA Mycorrhizal colonization of roots

Percent mycorrhizal root colonization was determined by the gridline intersect method outlined by Giovannetti and Mosse (1980).

Fresh root samples (weighing 300 mg) were cut into 1 cm pieces and placed in screw cap vials. The clearing of roots was achieved by treating them with 10 percent KOH (W/V) and heating them for 30 minutes at 90°C. The KOH solution was poured off and the roots were rinsed with tap water. Roots were then covered with 10 per cent HCl (v/v) for 10 minutes to neutralize the residual effect of alkali and to create an acidic medium required by the stain. Root bits were then stained with 0.05 per cent trypanblue in lactoglycerol. (lactic acid, glycerol and water in the ratio 40:40:20) by boiling them at 90°C for 30 minutes. Excess stain was decanted and the root samples were immersed in lactoglycerol for destaining (Phillips and Hayman, 1970).

The stained root bits were randomly placed on a grid plate of size 10 cm x 10 cm with 1 cm grid. The horizontal and verticle grid lines were scanned under a stereozoom microscope (model SZ1145 TR, Olympus, Japan) at 100x magnification to determine the total root bits and the root bits positive for VA Mycorrhizal colonization on grid line intersections.

The proportion of roots colonized by VAM was calculated by the formula

$$\text{VAM colonization (\%)} = \frac{\text{Total no. of intersections positive for VAM colonization}}{\text{Total number of intersections between roots and grid lines.}} \times 100$$

Total root length on the grid

The total root length in cm on the grid plate was calculated by the formula of Tennant (1975)

$$\text{Total Root length (cm)} = R \times \frac{N}{1.25}$$

Where R is grid size in cm and N is the total number of intersections between roots and grid lines.

Total mycorrhizal root length

The extent of root colonization estimated from gridline-intersect method was multiplied by the total root length to give the total root length colonized by VAM fungi in cm.

Dry weight of Plants

During 75 days growth period the defoliated leaves of each pot were collected and dried at 60°C in an oven. On the day of harvest after separating out the shoot portion, the roots were washed carefully under running tap water to remove adhering soil particles. The dry weights of root, shoot and defoliated leaves were recorded after drying them to a constant weight at 60°C. Total biomass of cowpea was computed by the summation of dry weights of shoot portion, root and defoliated leaves.

Phosphorus concentration in plant tissue

Plant P concentration was estimated colorimetrically by following the vanadomolybdate yellow colour method (Jackson, 1973).

The oven dried shoot and root samples were powdered in a grinder. The powdered plant samples (500mg) were digested using 10 ml of triacid mixture (nitric acid, perchloric acid and sulphuric acid in the ratio 6:3:1 v/v) in 100 ml conical flasks over a hot plate. The volume of the digested sample was made upto 100 ml in volumetric flasks using distilled water. 10 ml of this aliquot was taken and 10 ml of vandomolybdate reagent (Appendix II) was added. The volume was made up to 50 ml using distilled water. After shaking, this reaction mixture was allowed to stand for 20 minutes. The intensity of yellow colour due to phosphovanadomolybdate complex was read at 400 nm in a spectronic-20 spectrophotometer (Milton Roy). The phosphorus in the sample was determined by comparing with a standard curve developed using KH_2PO_4 as P source.

Statistical analysis

The data obtained from the pot culture experiment was subjected to analysis of variance by randomised complete block design using microvax system of Digital equipment corporation, USA (VAX/VMS version 5.4). Treatment means were separated by Duncan's Multiple Range (DMR) test (Little and Hills, 1978).

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The objective of this investigation was to know the possibility of producing VAM inoculum entrapped in alginate beads in different carrier materials, determining the number of I.P. and the survival of these propagules in alginate beads. An experiment was also carried out to study the response of an host plant to inoculation with alginate entrapped VAM inoculum.

Production of alginate entrapped VA Mycorrhizal inoculum

Carrier materials like perlite, soilrite, vermiculite, talc, kaolinite and bentonite were entrapped along with the VAM sand: soil inoculum to prepare alginate beads (Plates 1 to 8). The wet beads were globular in shape. The size of the alginate beads varied. This is because a sieve was used to pass the inoculum concoction into 0.1M $CaCl_2$ solution; which did not regulate the flow of the concoction. On drying the beads shrunk in size. The wet weight, dry weight and moisture content of the different carrier based VAM alginate beads are given in Table 1.

Perlite based dry alginate entrapped VAM beads weighing 5 g had the highest volume of 14.5 ml while the bentonite based dry VAM alginate beads which weighed the same had a volume of only 5.9 ml. The pH of the different carrier materials used in VAM alginate bead production varied from acidic to alkaline range. Perlite (7.35) and soilrite (7.15) had a pH which was around the neutral range (Table 2).

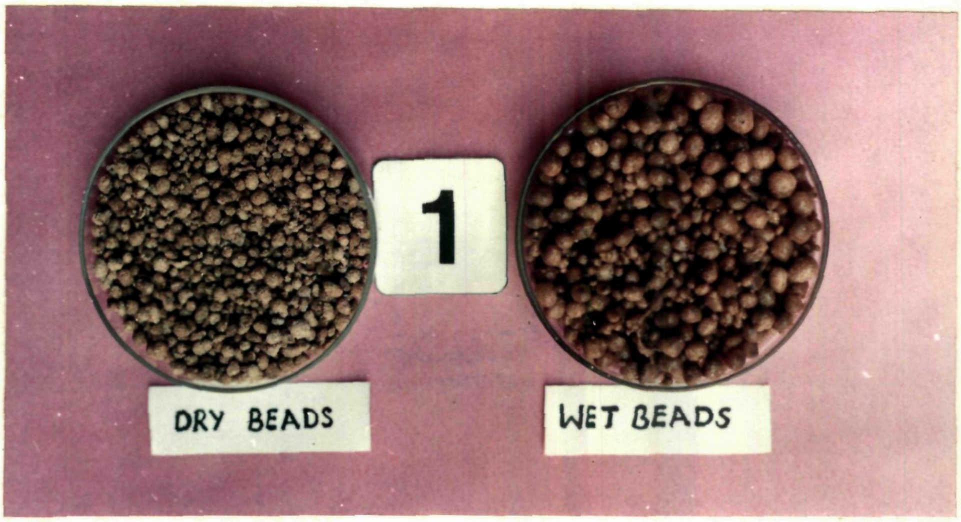


Plate 1. Perlite based alginate entrapped VA mycorrhizal inoculum

Plate 2. Soilrite based alginate entrapped VA mycorrhizal inoculum

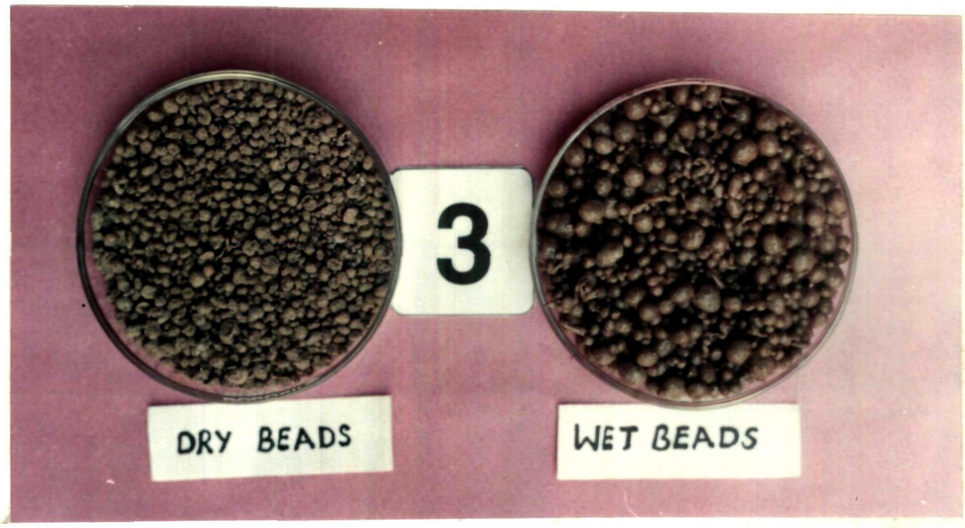


Plate 3. Vermiculite based alginate entrapped VA mycorrhizal inoculum

Plate 4. Talc based alginate entrapped VA mycorrhizal inoculum

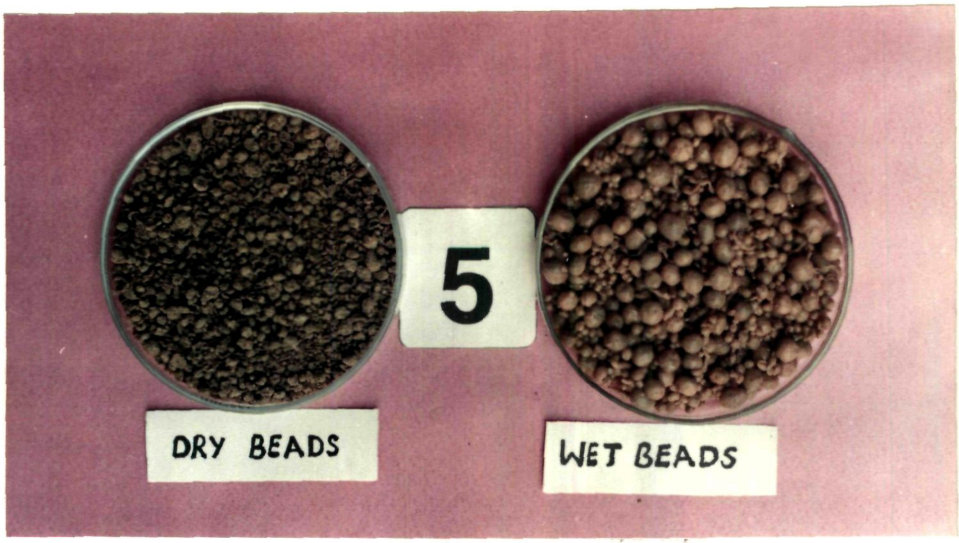


Plate 5. Kaolinite based alginate entrapped VA mycorrhizal inoculum

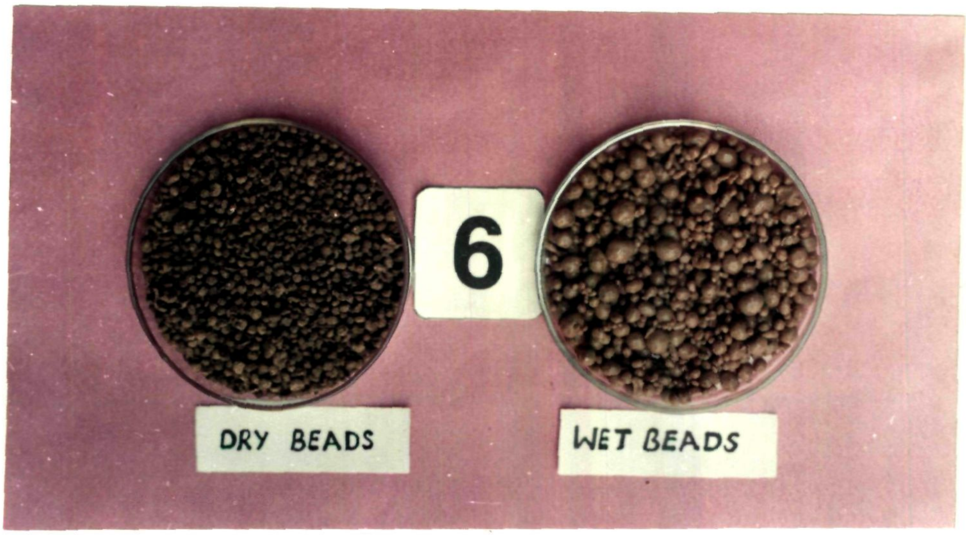


Plate 6. Bentonitebased alginate entrapped VA mycorrhizal inoculum

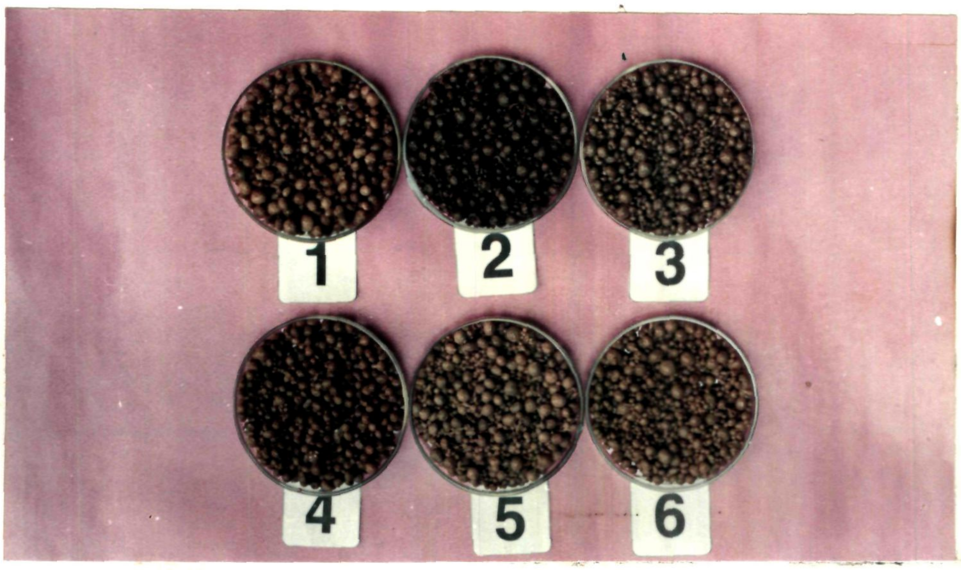


Plate 7. Different carrier based alginate entrapped VA mycorrhizal inocula (Wet beads)

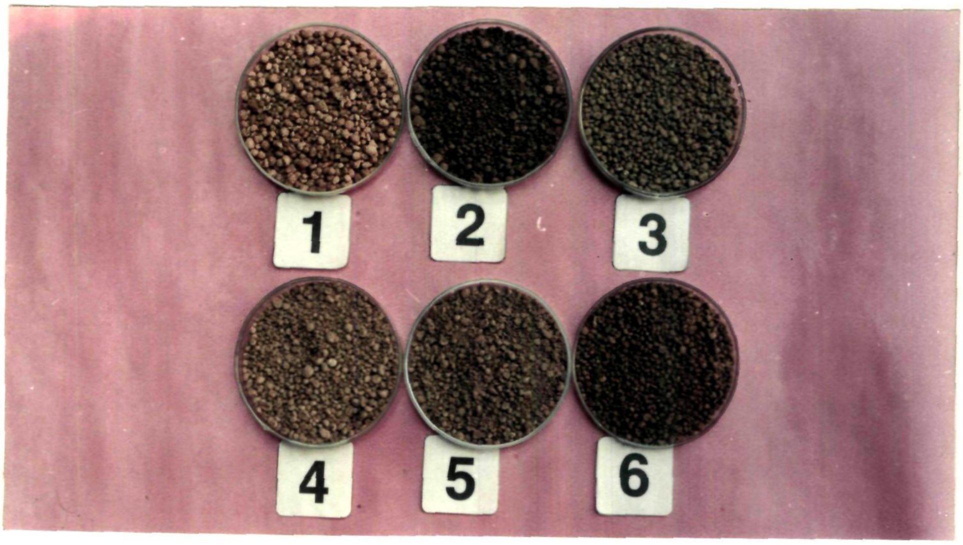


Plate 8. Different carrier based alginate entrapped VA mycorrhizal inocula (Dry beads)

Carrier materials 1. Perlite 2. Soilrite 3. Vermiculite
4. Talc 5. Kaolinite 6. Bentonite

Table 1.Wet weight, dry weight and moisture content of the different carrier based alginate entrapped VA mycorrhizal inocula

Carrier materials	Weight of beads (g)		Moisture content of wet beads (per cent)
	Wet beads*	dry beads**	
1 Perlite	76.44	18.70	81.30
2. Soilrite	74.28	18.60	81.40
3. Talc	76.03	17.16	81.84
4. Vermiculite	76.44	16.38	86.62
5. Kaolinite	76.24	17.60	82.40
6. Bentonite	79.81	18.77	81.23

* concoction containing 2 per cent sodium alginate, 2 per cent carrier 10 per cent sand:soil inoculum of *Glomus mosseae* and 100 ml sterile distilled water was used to prepare beads.

** weight obtained by drying 100 g wet carrier based VAM alginate beads.

Table 2. pH of the different carrier materials and volume of the different carrier based dry VA mycorrhizal beads

Carrier materials	pH	Volume of dry VAM beads (ml)
1 Perlite	7.35	14.5
2. Soilrite	7.15	8.2
3. Talc	7.90	6.1
4. Vermiculite	8.16	9.8
5. Kaolinite	5.62	6.0
6. Bentonite	5.55	5.9

Inoculum potential of the alginate entrapped VA mycorrhizal inoculum

The inoculum potential of the beaded inocula was estimated by determining the number of I.P. per unit weight by the MPN method using four fold dilution series. Each treatment had 11 dilutions from 4° to 4^{10} and 5 replications for each dilution. The 4° dilution was assumed to be positive in all the replications for all treatments. Therefore for each treatment 50 pine cell tubes with onion plants were observed for VAM root colonization. The sand : soil had the highest of 35 pine cell tubes which were positive for VAM root colonization. Among the wet beads, perlite based VAM alginate beads had the maximum number (26) of positive pine cells tubes for VAM root colonization. Bentonite and kaolinite based wet VAM alginate entrapped beads had the lowest of 21 tubes which were colonized by VAM fungi. The VAM I.P. number per g was 45.99 in sand : soil inoculum, the range being (21.52 to 98.30 per g of inoculum. The I.P. number of wet VAM alginate beads was maximum when perlite was used as a carrier material (3.79/g) followed by soilrite (2.87/g), talc (2.18/g), vermiculite (1.25/g), kaolinite and bentonite (0.95/g). Table 3 summarises the I.P. numbers of the wet alginate entrapped VAM beads.

The number of I.P. per unit weight present in dry alginate beads was computed using the data of VAM I.P. numbers present in wet beads and its moisture content. Perlite based dry VAM alginate beads had 20.28 I.P. per g of the inoculum which was highest, while the beads which had kaolinite as the carrier material contained 5.05 I.P. per g of the inoculum (Table 4). The I.P. number of the different carrier based wet and dry VAM beads is represented graphically in figure 1.

Table 3: VAM fungal (*Glomus mosseae*) infective propagule (I.P.) number in the different carrier based wet alginate entrapped VAM beads.

Carrier materials	No. of dilutions	No. of replications	No. of +ve tubes	I.P./g at 95% confidence level	
				range	mean
Perlite	11	5	26	1.78 to 8.10	3.79
Soilrite	11	5	25	1.35 to 6.14	2.87
Talc	11	5	24	1.02 to 4.65	2.18
Vermiculite	11	5	22	0.59 to 2.67	1.25
Kaolinite	11	5	21	0.44 to 2.03	0.95
Bentonite	11	5	21	0.44 to 2.03	0.95
Sand:soil inoculum	11	5	35	21.52 to 98.30	45.99

Table 4: VAM fungal (*Glomus mosseae*) infective propagule (I.P.) number in the different carrier based dry alginate entrapped VAM beads.

Carrier materials	I.P./g at 95% confidence level	
	range	mean
Perlite	9.49 to 43.33	20.28
Soilrite	7.98 to 36.42	17.04
Talc	5.94 to 27.12	12.69
Vermiculite	3.57 to 16.32	7.64
Kaolinite	2.36 to 10.79	5.05
Bentonite	2.52 to 11.51	5.39

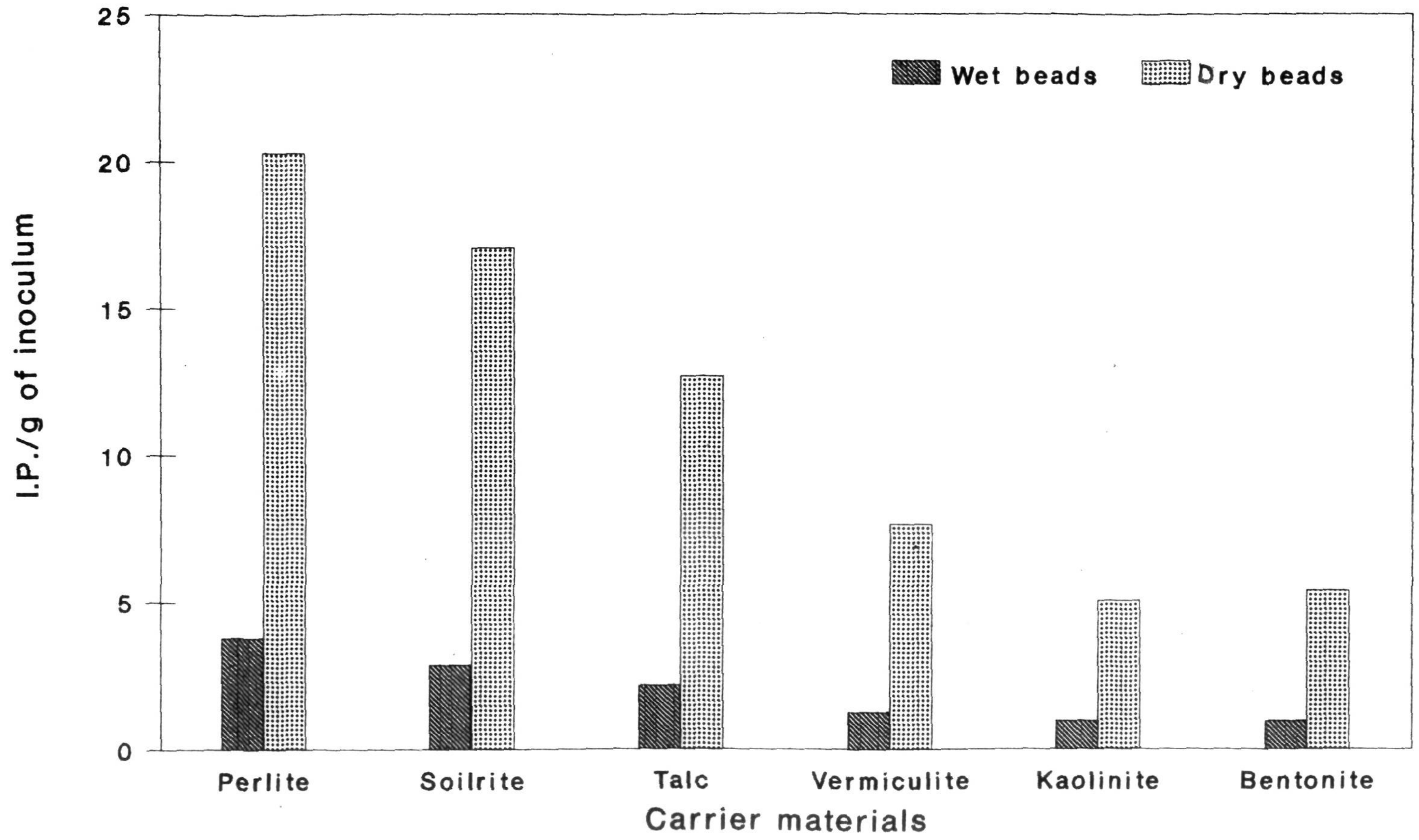


Fig 1: VAM fungal infective propagule number in the different carrier based wet and dry alginate entrapped VAM inocula

Viability of the alginate entrapped VA mycorrhizal beads

The survival of the VAM propagules in the different carrier based wet and dry alginate entrapped VAM inocula was studied for a period of six months using onion as the test plant. A decline in per cent root colonization was noticed as the days of storage increased. The decrease in per cent root colonization was more pronounced in dry alginate beads. Sand : Soil inoculum of VAM fungi induced the largest per cent root colonization in onion at different intervals of storage. Table 5 and figure 2 shows per cent root colonization in onion as influenced by inoculation of different carrier based VAM alginate beads stored for different intervals up to a period of 6 months.

Response of cowpea to inoculation with alginate entrapped VAM inoculum

Response of cowpea to inoculation with different alginate entrapped VA mycorrhizal inocula and sand : soil inoculum was studied. The plant and mycorrhizal parameters studied are given below.

Plant height

The height of cowpea increased steadily from the day of planting to the day of harvest (75 days after planting) in all the treatments (Table 6). Vermiculite based dry alginate entrapped VAM inoculation resulted in the highest plant height of 77.20 cm on the day of harvest. There was no significant difference in plant height between the uninoculated control soilrite vermiculite and talc based wet VAM alginate beads inoculated plants.

Number of trifoliate leaves per plant

The number of fully opened trifoliate leaves was around one on 20th

Table 5: Per cent root colonization of onion as influenced by inoculation with different carrier based alginate entrapped VAM inoculum stored for different intervals.

Treatments* (Carrier materials)	Percent root colonization after different days of storage					
	30	60	90	120	150	180
A. Wet beads						
1. Perlite	39.57	36.10	28.64	30.21	28.90	24.82
2. Soilrite	36.54	25.81	32.90	27.76	22.83	25.95
3. Talc	32.59	37.99	27.16	23.81	23.46	22.76
4. Vermiculite	34.26	27.49	29.64	24.85	23.69	26.92
5. Kaolinite	32.94	26.37	25.34	28.93	23.39	20.10
6. Bentonite	29.30	28.09	25.60	17.31	29.80	19.89
B. Dry beads						
7. Perlite	33.51	26.49	25.58	27.74	16.22	19.14
8. Soilrite	32.16	28.05	25.67	21.59	20.85	18.19
9. Talc	32.94	33.09	20.17	20.07	21.63	12.44
10. Vermiculite	31.34	29.92	24.79	26.47	17.68	16.95
11. Kaolinite	28.25	22.05	21.80	23.05	19.63	13.63
12. Bentonite	28.39	26.56	21.20	19.25	19.72	14.89
13. Sand soil inoculum	48.53	48.10	45.91	40.02	37.92	33.16

* 5 g of the different inocula was added per replication

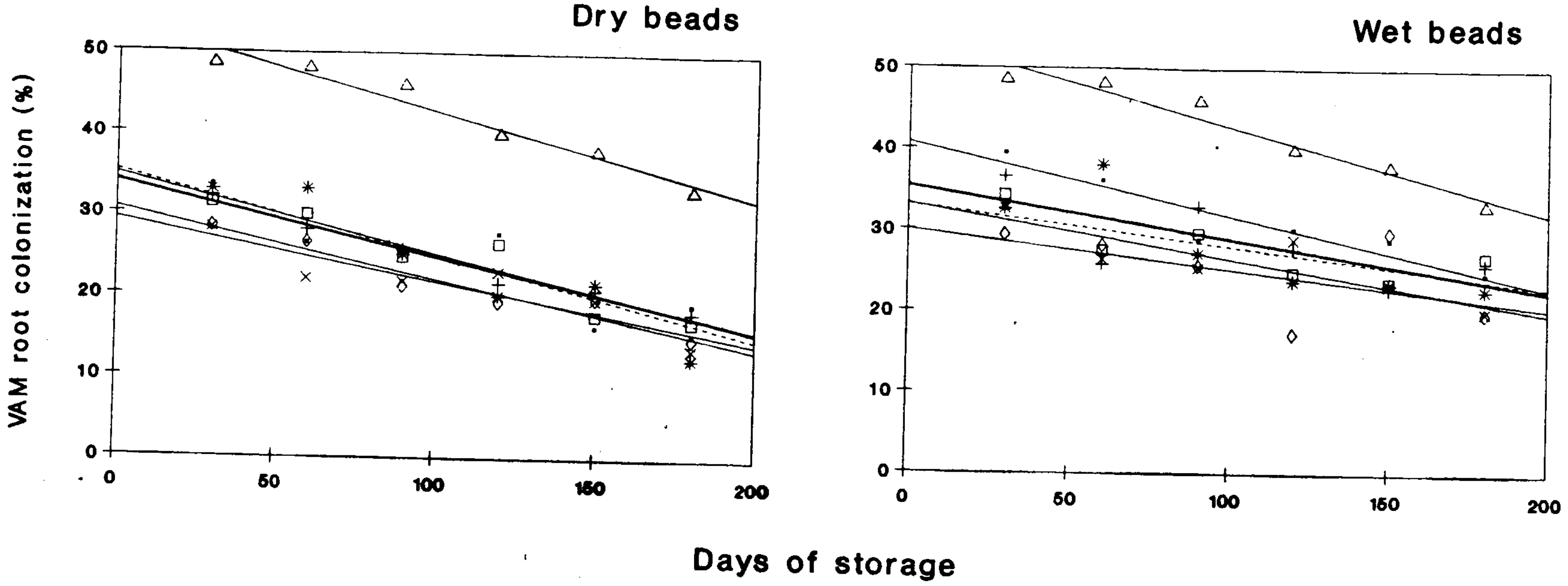


Fig 2: Per cent root colonization of onion as influenced by inoculation with different carrier based alginate entrapped VAM inocula stored for different intervals

Table 6. Effect of inoculation with different carrier based alginate entrapped VAM inocula on height of cowpea.

Treatments* (Carrier materials)	Plant height in centimetre at different days after planting 20	40	60	75
A. <u>Wet beads</u>				
1. Perlite	18.05^{ab}	19.00^{b}	35.75^{bcde}	68.50^{abc}
2. Soilrite	19.90^{a}	20.90^{ab}	38.80^{ab}	59.60^{cd}
3. Talc	18.15^{ab}	20.10^{ab}	35.65^{bcde}	52.80^{d}
4. Vermiculite	17.33^{b}	19.30^{ab}	30.60^{e}	61.30^{bcd}
B <u>Dry beads</u>				
5. Perlite	19.55^{ab}	20.55^{ab}	38.15^{abc}	72.90^{ab}
6. Soilrite	19.55^{ab}	20.90^{ab}	36.35^{abcde}	76.20^{a}
7. Talc	18.95^{ab}	20.95^{ab}	32.45^{cde}	69.10^{abc}
8. Vermiculite	19.60^{ab}	22.00^{a}	42.20^{a}	77.20^{a}
9. Sand soil inoculum	19.20^{ab}	20.55^{ab}	36.90^{abcd}	69.10^{abc}
10. Control	18.95^{ab}	20.60^{ab}	31.25^{de}	52.20^{d}

Means with the same superscript do not differ significantly at $P=0.05$ level by Duncan's multiple range test.

* Treatments 1 to 9 contained 500 I.P. of *Glomus mosseae* per pot (2 plants)

day after planting and increased to maximum of 10.30 leaves per plant after 75 days after planting. (table 7). There was a striking difference in the number of leaves between the control plants which received no VAM inoculation (7.00 leaves/plant) and the plants which received sand : soil or vermiculite based dry VAM alginate beads (10.30 leaves/plant). The wet VAM alginate beads with perlite and vermiculite as carriers and the dry VAM alginate beads with perlite, soilrite and talc as carriers, when inoculated to pots, resulted in around 9 leaves per plant.

VAM colonization of roots

Plants grown in control pots which had the indigenous mycorrhizal population had the lowest per cent root colonization of 61.95. The highest level of VAM colonization (76.25%) was noticed in plants grown in soil inoculated with VAM sand : soil inoculum. Inoculation of alginate entrapped VAM inoculum to soil resulted in VAM root colonization which varied between 70.91 per cent (perlite based wet VAM beads) to 66.27 per cent (vermiculite based wet VAM beads).

Total root length (cm) is the length of roots which were spread on the grid line plate for estimating the per cent root colonization of VAM fungi. The root length colonized by VAM fungi is another way of depicting the infectivity of the mycorrhizal inoculum. The length of root colonized by VAM fungi was longest in plants inoculated with perlite based wet VAM beads (table 8 and figure 3).

Root dry weight

The maximum root biomass was produced by plants in soils which were inoculated with sand : soil inoculum (0.569 g). However this value was

Table 7: Effect of inoculation with different carrier based alginateentrapped VAM inocula on number of leaves of cowpea.

Treatments* (Carrier materials)	No. of fully opened trifoliate leaves per plant at different days after planting			
	20	40	60	75
A. <u>Wet beads</u>				
1. Perlite	1.00^{b}	1.90^{a}	6.10^{ab}	9.30^{a}
2. Soilrite	1.30^{a}	1.80^{a}	5.60^{ab}	8.60^{abc}
3. Talc	1.00^{b}	1.80^{a}	5.70^{ab}	7.30^{bc}
4. Vermiculite	1.00^{b}	1.70^{a}	6.10^{ab}	9.20^{a}
B <u>Dry beads</u>				
5. Perlite	1.10^{ab}	1.90^{a}	5.60^{ab}	9.50^{a}
6. Soilrite	1.00^{b}	1.80^{a}	5.90^{ab}	9.00^{ab}
7. Talc	1.10^{ab}	1.90^{a}	5.30^{ab}	9.40^{a}
8. Vermiculite	1.10^{ab}	2.00^{a}	5.60^{ab}	10.30^{a}
9. Sand soil inoculum	1.10^{ab}	1.90^{a}	6.30^{b}	10.30^{a}
10. Control	1.00^{b}	1.60^{a}	4.80^{b}	7.00^{c}

Means with the same superscript do not differ significantly at P=0.05 level by Duncan's multiple range test.

*Treatments 1 to 9 contained 500 I.P. of *Glomus mosseae* per pot (2 plants)

Table 8: Effect of inoculation of different carrier based alginate entrapped VAM inocula on percent mycorrhizal root colonization of cowpea

Treatments* (Carrier materials)	VAM root colonization (per cent)	Total root length (cm)	VAM root length (cm)
A. <u>Wet beads</u>			
1. Perlite	70.91^{ab}	129.28	91.67
2. Soilrite	68.30^{b}	114.40	78.14
3. Talc	69.91^{b}	125.12	87.47
4. Vermiculite	66.27^{bc}	114.72	76.03
B <u>Dry beads</u>			
5. Perlite	68.40^{b}	123.36	84.38
6. Soilrite	67.99^{b}	107.20	72.89
7. Talc	69.59^{b}	119.20	82.95
8. Vermiculite	70.78^{ab}	114.72	84.37
9. Sand soil inoculum	76.25^{a}	116.64	88.94
10. Control	61.95^{c}	135.36	83.86

Means with the same superscript do not differ significantly at P=0.05 level by Duncan's multiple range test.

* Treatments 1 to 9 contained 500 I.P. of *Glomus mosseae*/pot (2 plants)

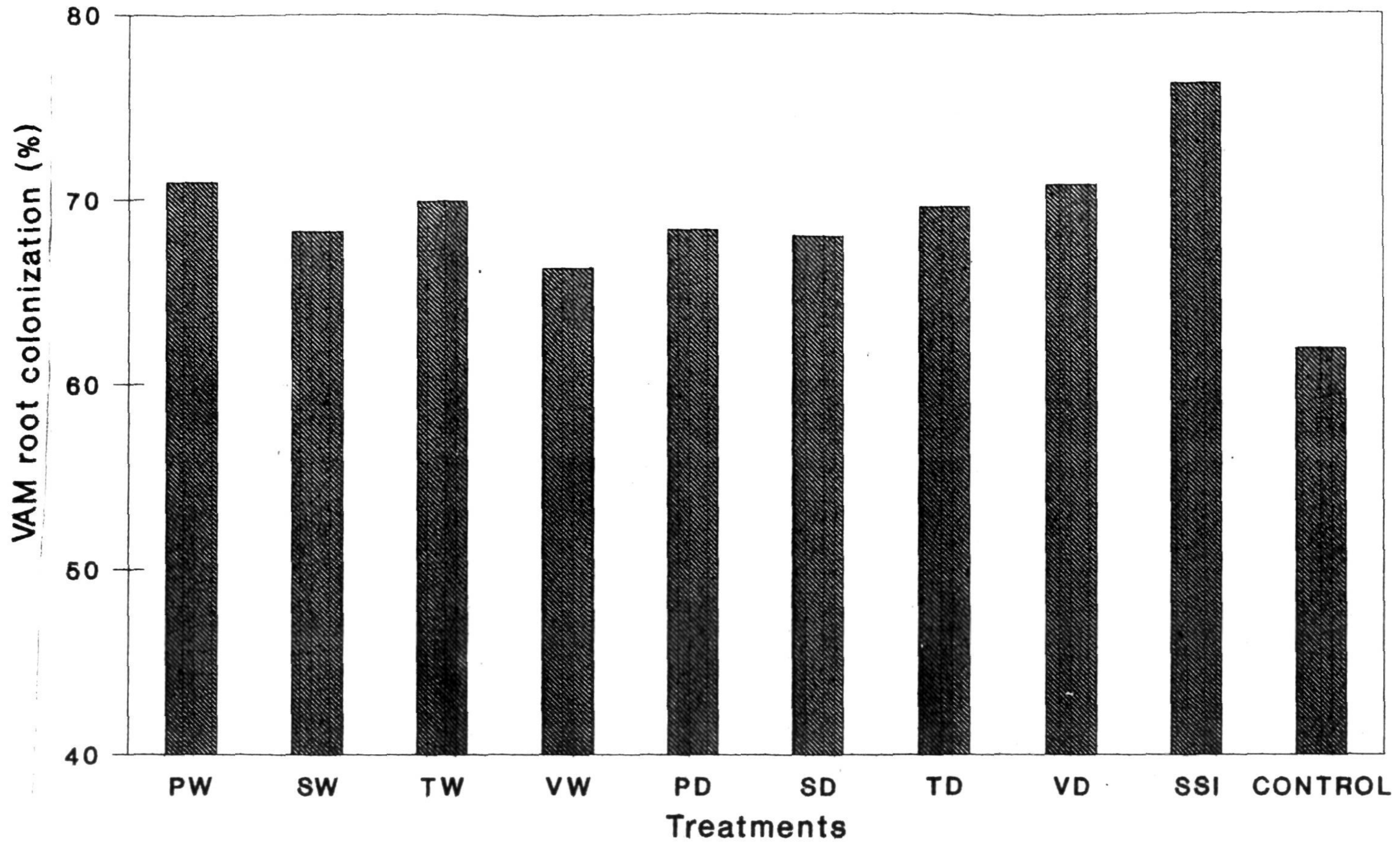


Fig 3: Effect of inoculation of different carrier based alginate entrapped VAM inocula on percent mycorrhizal root colonization of cowpea

P-Perlite, S-Soilrite, T-Talc, V-Vermiculite, SSI-Sand Soil Inoculum,
W-Wet VAM alginate beads; D-Dry VAM alginate beads

not significantly different from those of plants in soils inoculated with with wet alginate beads which had talc and perlite as carrier materials and dry alginate beads with perlite and soilrite as carrier materials. Uninoculated control plants had the least root biomass of 0.489 g (table 9 and figure 4).

Shoot dry weight

Shoot dry weight included the dry weight of stem, leaves and defoliated leaves. Uninoculated plants had 1.397 g of dry shoot biomass which was the least. The dry shoot weight (g) of the plants grown in soil inoculated with sand:soil inoculum (1.977 g), vermiculite (1.923 g), soilrite (1.732 g), perlite (1.705 g) and talc (1.675 g) based dry alginate beads and soilrite based wet VAM alginate beads (1.679 g) did not differ significantly (table 9 and figure 4).

Total biomass

There was a significant increase in biomass between the plants grown uninoculated and VAM inoculated soil (table 9 and figure 4). The soil inoculated with sand:soil inoculum, dry VAM alginate beads with carriers perlite, soilrite talc and vermiculite and wet alginate entrapped VAM inoculum with carriers perlite and soilrite produced plants whose biomass was statistically on par with each other. The uninoculated plants had a biomass of 1.866 g which was the least when compared to the other treatments.

Phosphorus concentration of root

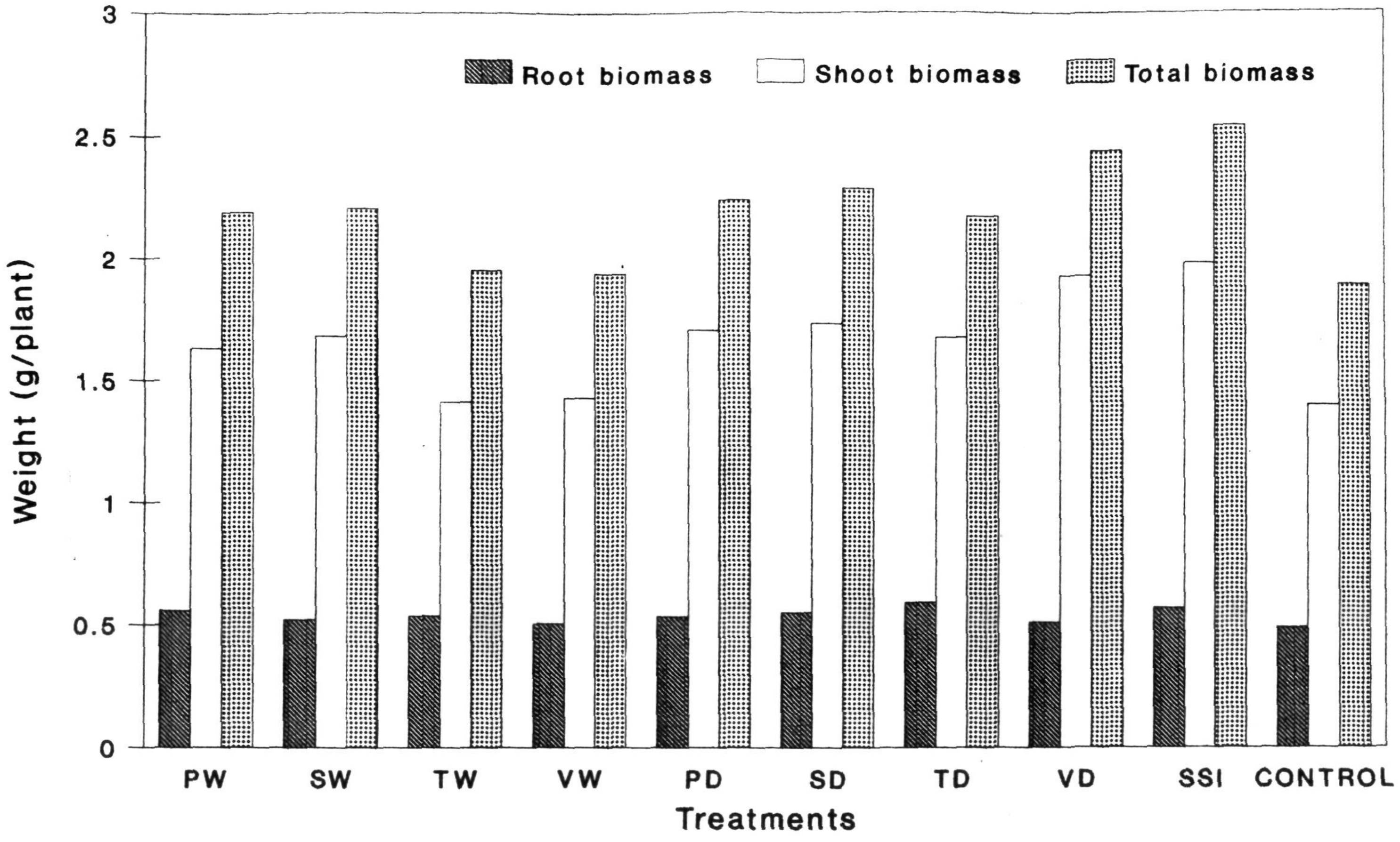
Inoculation of soil with dry VAM soilrite alginate beads yielded plants with 0.248 per cent P in roots, which was the highest. And this value did not differ significantly from the plants grown in soil inoculated with sand:soil inoculum (0.229%) dry VAM alginate beads with perlite (0.246 %) talc (0.237 %) and vermiculite (0.233 %) as carriers. The least per cent P concentration in roots was

Table 9: Effect of inoculation with different carrier based alginate entrapped VAM inocula on root, shoot and total biomass of cowpea

Treatments* (Carrier materials)	root dry weight (g/plant)	shoot dry weight (g/plant)	Total biomass (g/plant)
A. <u>Wet beads</u>			
1 Perlite	0.558^{ab}	1.628^{bcd}	2.186^{abc}
2 Soilrite	0.523^{bcd}	1.679^{abcd}	2.202^{abc}
3 Talc	0.537^{abc}	1.412^{cd}	1.949^{bcd}
4 Vermiculite	0.506^{cd}	1.425^{cd}	1.931^{cd}
B <u>Dry beads</u>			
5 Perlite	0.534^{abc}	1.705^{abc}	2.239^{ab}
6 Soilrite	0.552^{ab}	1.732^{ab}	2.284^{a}
7 Talc	0.493^{d}	1.675^{abcd}	2.168^{abcd}
8 Vermiculite	0.511^{cd}	1.923^{a}	2.434^{a}
9 Sand : Soil Inoculum	0.569^{a}	1.977^{a}	2.539^{a}
10 Control	0.489^{d}	1.397^{d}	1.886^{d}

Means with the same superscript do not differ significantly at P=0.05 level by Duncan's multiple range test.

*Treatments 1 to 9 contained 500 I.P. of *Glomus mosseae*/pot (2 plants)



. Fig 4: Effect of inoculation of different carrier based alginate entrapped VAM inocula on dry weight of root, shoot and total biomass of cowpea

P-Perlite, S-Soilrite, T-Talc, V-Vermiculite, SSI-Sand Soil Inoculum,
W-Wet VAM alginate beads; D-Dry VAM alginate beads

present in control plants (0.181 %) (table 10 and figure 5).

Phosphorus concentration of shoot

Control plants had the least phosphorus concentration in the shoot of 0.150 per cent while the soil which was inoculated with perlite based wet VAM alginate beads produced plants which had the highest of 0.192 per cent phosphorus in shoot (table 10 and figure 5). Even though VAM inoculation (either as carrier based alginate entrapped VAM inoculum or as sand:soil inoculum) to soil caused an increase in P concentration of plant, the difference noticed was not significant.

Phosphorus content of root.

The phosphorus content (mg/plant) of roots was highest in plants grown in soil inoculated with dry soilrite based VAM alginate beads (1.369 mgP/plant). Soils which were not inoculated had plants with the least (0.885 mgP/plant) root phosphorus content. Further the difference in phosphorus content of root between plants grown in soil inoculated with sand:soil inoculum of *Glomus mosseae* and dry alginate entrapped VAM beads with perlite, soilrite, talc and vermiculite as carriers were not significant (table 11 and figure 6).

Phosphorus content of shoot

Significant differences were observed between the phosphorus content (mgP/plant) of shoot in uninoculated plants and plants inoculated with VA mycorrhizal fungi. Uninoculated control plants had the least amount of 2.096 mg phosphorus per plant. Soil inoculated with sand : soil VAM inoculum had plants with the highest amount of phosphorus content in shoot (3.302 mgP/plant) which was on par with the plant in soil inoculated

Table 10: Effect of inoculation with different carrier based alginate entrapped VAM inocula on percent Phosphorus concentration in root and shoot biomass of cowpea.

Treatments (Carrier materials)	P concentration (per cent) root	shoot
A. Wet beads		
1 Perlite	0.194^{cd}	0.192^{ab}
2 Soilrite	0.186^{d}	0.166^{ab}
3 Talc	0.202^{bcd}	0.166^{ab}
4 Vermiculite	0.186^{d}	0.164^{ab}
B Dry beads		
5 Perlite	0.246^{a}	0.162^{ab}
6 Soilrite	0.248^{a}	0.166^{ab}
7 Talc	0.237^{ab}	0.173^{ab}
8 Vermiculite	0.233^{ab}	0.163^{a}
9 Sand : Soil Inoculum	0.229^{abc}	0.167^{ab}
10 Control	0.181^{d}	0.150^{b}

Means with the same superscript do not differ significantly at P=0.05 level by Duncan's multiple range test.

*Treatments 1 to 9 contained 500 I.P. of *Glomus mosseae*/ pot (2 plants)

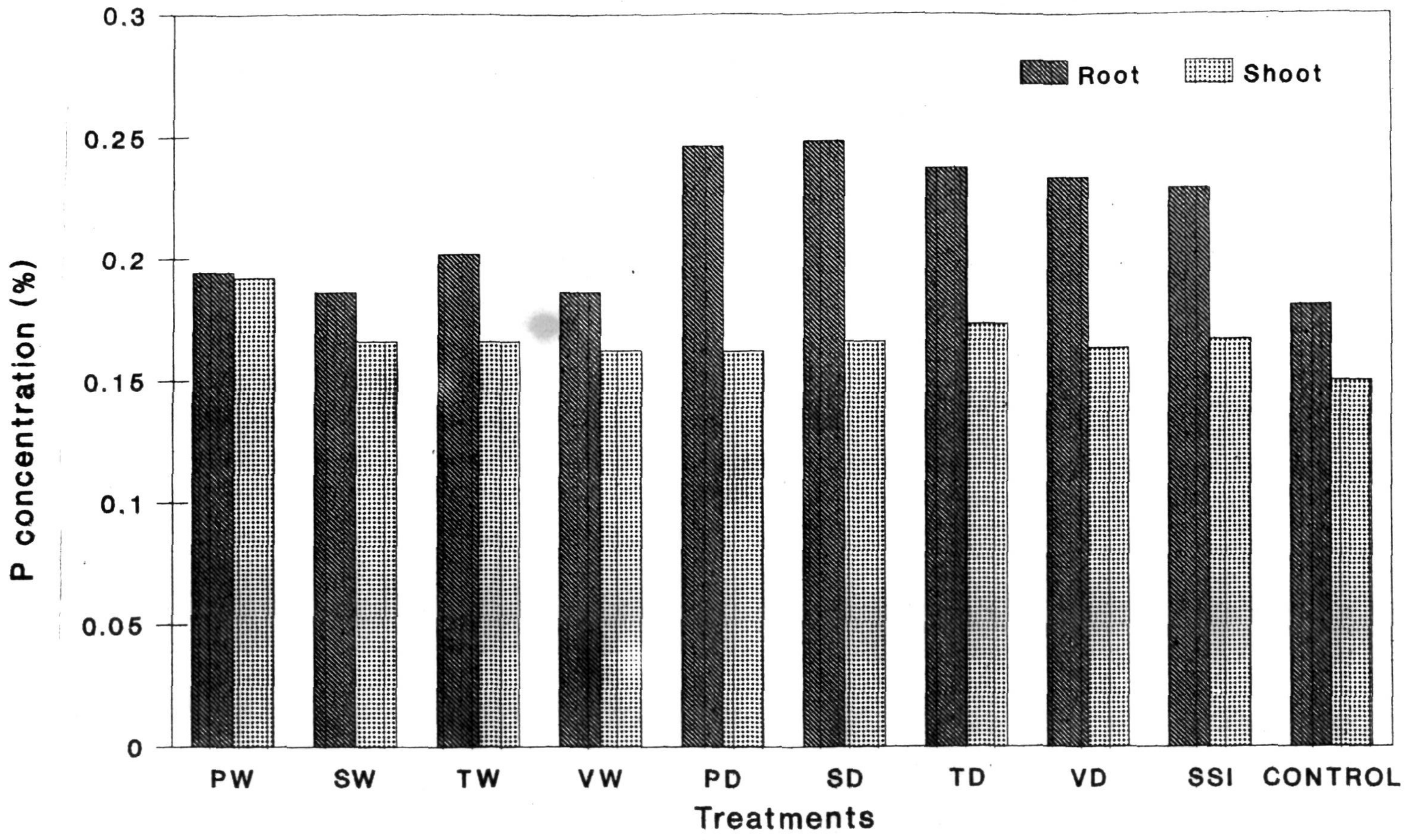


Fig 5: Effect of inoculation of different carrier based alginate entrapped VAM inocula on percent Phosphorus concentration in root and shoot of cowpea

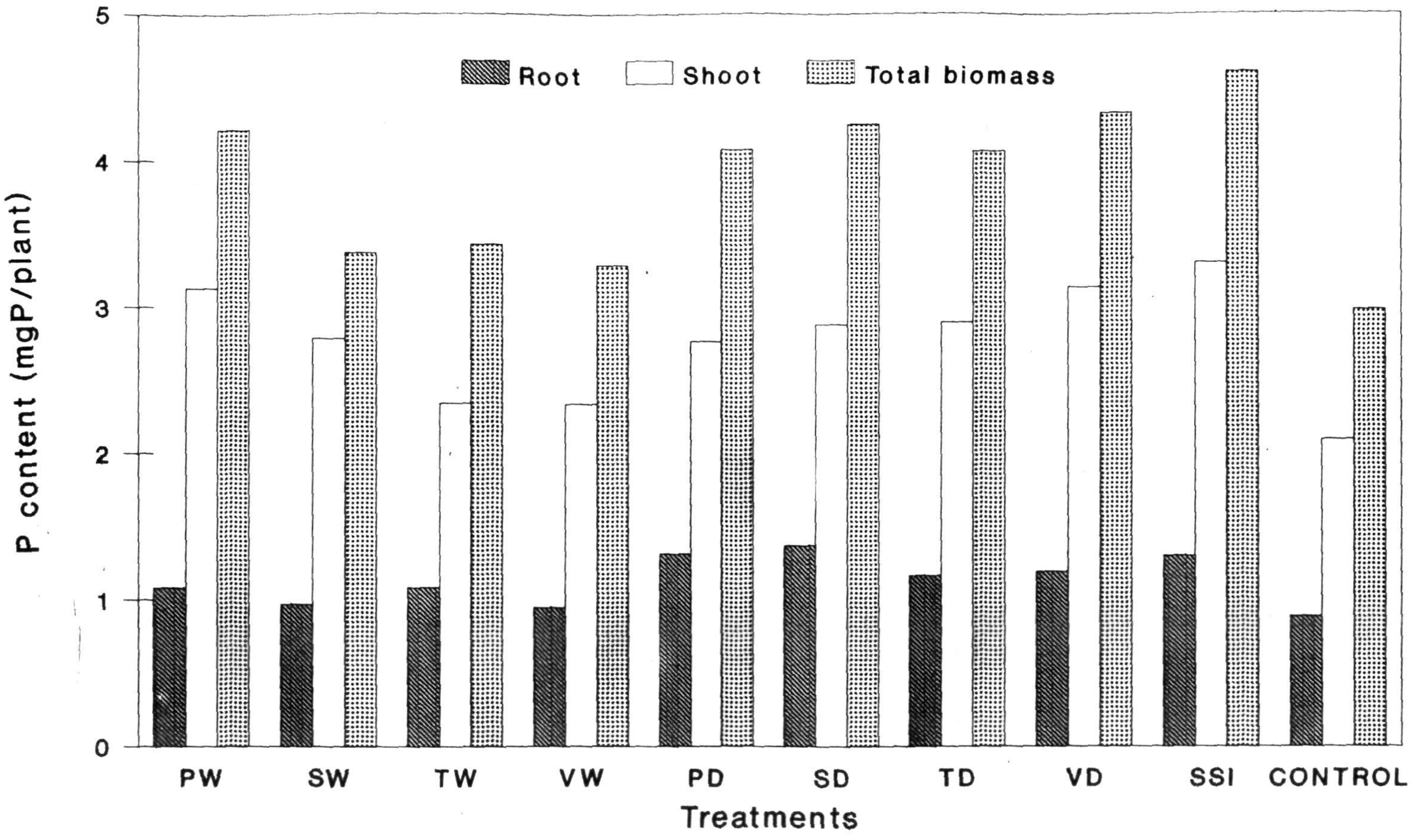
P-Perlite, S-Soilrite, T-Talc, V-Vermiculite, SSI-Sand Soil Inoculum,
W-Wet VAM alginate beads; D-Dry VAM alginate beads

Table 11. Effect of inoculation with different carrier based alginate entrapped VAM inocula on Phosphorus content of root, shoot and total biomass of cowpea.

Treatments * (Carrier materials)	P content in root (mg/plant)	P content in shoot (mg/plant)	Total P content in plant (mg / plant)
A. <u>Wet beads</u>			
1 Perlite	1.083 bcd	3.126 a	4.209 ab
2 Soilrite	0.973 cd	2.787 ab	3.760 bc
3 Talc	1.085 bcd	2.344 bc	3.429 cd
4 Vermiculite	0.941 cd	2.337 bc	3.278 cd
B <u>Dry beads</u>			
5 Perlite	1.312 a	2.762 ab	4.074 ab
6 Soilrite	1.369 a	2.875 ab	4.244 ab
7 Talc	1.168 abc	2.898 ab	4.066 ab
8 Vermiculite	1.191 abc	3.135 a	4.326 ab
9 Sand : soil Inoculum	1.303 ab	3.302 a	4.605 a
10 Control	0.885 d	2.096 c	2.981 d

Means with the same superscript do not differ significantly at P=0.05 level by Duncan's multiple range test.

*Treatments 1 to 9 contained 500 I.P. of *Glomus mosseae*/ pot (2 plants)



.Fig 6: Effect of inoculation of different carrier based alginate entrapped VAM inocula on phosphorus content of root, shoot and total biomass of cowpea

P-Perlite, S-Soilrite, T-Talc, V-Vermiculite, SSI-Sand Soil Inoculum,
W-Wet VAM alginate beads; D-Dry VAM alginate beads

with perlite and soilrite as carrier in wet beads and vermiculite, talc, soilrite and perlite as carrier in dry beads (table 11 and figure 6).

Total phosphorus content of plant.

Inoculation with mycorrhizal fungi increased the phosphorus content of the plant (table 11 and figure 6). Plants grown in soil inoculated with sand:soil inoculum of VAM fungi exhibited highest total phosphorus content (4.605 mgP/plant). The uninoculated soil produced plants which had only 2.981 mg phosphorus per plant. The total phosphorus content of plants in soil inoculated with sand:soil inoculum, dry VAM alginate beads with the four different carrier materials (perlite, soilrite, talc and vermiculite) wet VAM alginate beads with perlite as carrier, were not statistically significant.

DISCUSSION

V. DISCUSSION

The results of the investigation titled 'Production of alginate entrapped VA mycorrhizal inoculum' presented in the previous chapter are discussed here in the light of the available literature.

Production of alginate entrapped VA mycorrhizal inoculum

Alginate is a polysaccharide made up of ß.D mannuronate and _-L guluronate. In the presence of metal ions, Ca^{2+} /Ba^{2+} /Al^{3+} / Cu^{2+} an aqueous solution of alginate forms a gel by ionic cross linking (ionotropic gelation) (Brodelius and Vandamme, 1987). Thus by adding a concoction containing sodium alginate (2%), carrier material (2%) and sand : soil VAM inoculum (10%) dropwise into 0.1M $CaCl_2$ solution beads are formed. This process entraps the carrier material and sand : soil VAM inoculum in the sodium alginate beads. Perlite, kaolinite and bentonite have been tried earlier as carrier materials for immobilization of microbial inoculants in sodium alginate beads. Talc, vermiculite and soilrite were tried for the first time in this study. (Plates 1 - 8).

The reaction of an aqueous solution of sodium alginate with certain metal ions such as Ca^{2+} are often used to formulate rhizobial inoculum (Jung *et al.*, 1982; Hegde and Brahmaprakash, 1992), *Azospirillium* and *Pseudomonas* inocula (Bashan, 1986), *Nitrosomonas europea* inoculum (Van Ginkel *et al.*, 1983), potential biocontrol agents (Fravel *et al.*, 1985), ectomycorrhizal inoculum (Kropacek *et al.*, 1989; Cudlin *et al.*, 1992), mushroom mycelium (Romaine and Schlagnhaufer, 1992) and pesticides (Debongnie and Pussmier, 1992; Pussemier *et al.*, 1992). Immobilization by entrapment method (ionotrophic gelation) is also used in the production of synthetic seeds. Immobilization of carrot somatic embryo together with hot water extract of marine cyanobacterium *Synechococcus* sp in alginate beads resulted in enhanced germination of the artificial seeds (Wake *et al.*, 1992). Duponnois and Garbaye (1991) inoculated Douglas fir with ectomycorrhizal fungus *Laccaria laccata* and mycorrhization helper bacteria. A dual inoculum made of calcium alginate beads containing the two microorganisms appeared suitable for inoculating forest nurseries. Strullu and Plenchette (1991) successfully entrapped intraradical forms of VAM fungus, *Glomus mosseae* and VAM root pieces in calcium alginate beads. Leek plants inoculated with these beads developed VA mycorrhizal associations.

Inoculum potential of alginate entrapped VA mycorrhizal inoculum.

In the present study the VAM infective propagule numbers of the different carrier (perlite, soilrite, vermiculite, talc, kaolinite, bentonite) based alginate entrapped VAM inoculum was estimated by the MPN method using four fold dilutions (Sieverding, 1991). The MPN bioassay detects all infective propagules of VAM capable of infecting the assay host. It does not enumerate non-viable spores (Porter 1979, An *et al.*, 1990). The MPN method is relatively easy to conduct though it requires some space and time (6-8 weeks). A suitable test plant can be used to test the MPN. In this study onion was used as a test plant. Onion is highly mycorrhizal and it is relatively easy to stain its roots and to observe for mycorrhizal colonization. The I.P. number per unit weight of the inoculum can be estimated by the MPN method, additionally the limits of confidence of the value can also be computed (Sieverding, 1991). Error in the MPN method can be minimized by using a low dilution factor and a large number of replications (Cochran, 1950).

Among the carrier based wet alginate beads, perlite - alginate beads had the highest I.P. numbers of 3.79 per g. of inoculum (Table 3). Perlite has been used earlier as a carrier material in alginate beads for *Rhizobium* inoculum (Hegde and Brahmaprakash, 1992) and ectomycorrhizal inoculum (Kropacek *et al.*, 1989; Cudlin *et al.*, 1992). Alginate is a relatively inert substance and it offers a mild condition for entrapment (Brodelius and Vandamme, 1987). Perlite is also a relatively inert material and it is very light in weight. The pH of perlite is 7.35. Five grams of perlite based dry alginate beads had a volume of 14.5 ml which was the maximum among the different carrier based beads (Table 2). The spore germination and effectiveness of *Glomus mosseae* is dependent on pH. *Glomus mosseae* spores can germinate well on water agar or soil extract agar at pH 6 to 9 (Gerdemann and Trappe, 1974). The VAM fungus *Glomus mosseae* shows 60 per cent effectiveness at pH 6 and 8 and nearly 100 per cent effectiveness at neutral pH (Sieverding, 1991). The favourable pH and better aeration provided to the VAM fungus by the carrier is probably the reason for the survival of VAM propagules in perlite - alginate VAM beads. Soilrite ranked second for the number of VAM fungal I.P. present in wet alginate beads. Soilrite is a mixture of peat moss, perlite and expanded exfoliated vermiculite in the proportion 1/3 : 1/3 : 1/3 by volume. With its optimum pH of 7.15, the soilrite based wet VAM alginate beads contained 2.87 I.P per g. of the inoculum.

The wet VAM alginate beads having talc, vermiculite, kaolinite and bentonite as carriers followed in order or the number of VAM I.P. they contained. Kaolinite clay was added to improve the survival of *Rhizobium* in alginate beads by Jung *et al.* (1982). A mixed response was got with *Rhizobium* sp. strain 135 sp 2 deriving the protective benefit of clay while the strain 138 did not benefit by clay addition. Bushby and Marshall (1977) also observed that montmorillonite clay protected some rhizobial strains only. Among the different biocontrol agents encapsulated in alginate - clay (pyrophillite, hydrous aluminium silicate) matrix all fungi like *Talaromyces flavus, Gleocladium virens, Pencillium oxalicum* and *Trichoderma viride* were viable after pellet formation, but not *Pseudomonas cepacia*. (Fravel *et al.*, 1985). VAM intraradical forms (vesicles and mycelial fragments) immobilized in calcium alginate beads did not lose their ability to infect roots even after one month of storage at 4°C. (Strullu and Plenchette, 1992). Strullu *et al.*, (1989) suggests that calcium may be important for the regeneration of intraradical forms of VAM fungi.

The I.P. number of *Glomus mosseae* in sand : soil inoculum was 45.99 per g of the inoculum. Considering that 100 ml of the concoction contained 10 g of the sand : soil inoculum, the VAM I.P. number approximately in wet beads should have been a tenth of the sand : soil inoculum i.e. around 4.5 per g of wet beads. The less number of I.P. in the wet VAM alginate beads (Table 3) indicate that a portion of the VAM propagules might have been lost during the entrapment or solubilization process. The variation in I.P. of VAM fungi among the different carrier based VAM alginate beads could be attributed to the characteristics of the carrier materials used for entrapment purpose. The inoculum potential of the dry VAM alginate beads was not determined experimentally. This value was computed by using the VAM fungal I.P. numbers of different carrier based wet alginate beads and its moisture content. Hence the MPN value of the different carrier based dry VAM alginate beads were higher and the trend of the inoculum potential of the different carrier based wet VAM alginate beads was also seen in dry beads (Table 4).

Tommerup (1992) reported that the success of the MPN method vary from good to negligible. Probabilities but not actual numbers are measured here. Physical, chemical and biological factors affect the MPN experiment. Temperature, time of harvest of bioassay host (Wilson and Trinick, 1982), host plant and growth medium (Sylivia and Burks, 1988) all affect the reliability of the MPN technique to determine VAM fungal I.P. number. In the MPN method assumptions involved are that, the propagules are randomly distributed, the dilution is proportional to the number of propagules and the organism is detected by the assay method (Wilson and Trinick, 1982; Tommerup, 1992). Wilson and Trinick (1982) point out that the MPN method provides only a relative measure of the density of VAM propagules, hence an absolute value is not obtained. Furthermore, the MPN technique is labour intensive and time consuming.

Viability of the alginate entrapped VAM inoculum

The propagules of VAM were viable in the wet as well as dry VAM alginate beads for 6 months and could colonize onion roots (as done in present study), perhaps it is viable for a longer period. However the percent VAM root colonization decreased as the age of the beaded inocula increased (Table 5). Similar results were obtained by the immobilization of bacterial inoculants in calcium alginate. Air drying of beads prevented the multiplication of contaminants, however it drastically reduced the number of living bacterial cells in the alginate beads (Jung *et al.*, 1982; Bashan, 1986). Wilson and Trinick (1982) there was a reduction in the MPN value of different VAM fungi in sand : soil inoculum as it ages. This is probably because sand : soil inoculum consists mainly of spores, the hyphae being dead. A decrease in the viability of the spores and death of VAM fungal hyphae as the inoculum ages lead to the decrease in MPN value and also per cent root colonization.

Response of cowpea to inoculation with alginate entrapped VAM inoculum.

The alginate entrapped VAM beads successfully colonized the roots of cowpea on inoculation (Table 6). However the percent VAM root colonization was highest in plants which received sand : soil inoculum. This is probably because the propagules are released at a slower rate from calcium alginate beads, as the alginate beads have to be degraded first for the inoculum to be released to infect and colonize roots. This upholds the view expressed by Bashan (1986) and Hegde and Brahmaprakash (1992), working with *Azosprillium* and *Rhizobium* entrapped in alginate beads that bacteria are released at a slower rate from alginate beads compared to peat based inoculum. Intraradical forms and infected root pieces of VAM fungus (*Glomus* sp.) entrapped in calcium alginate beads successfully developed mycorrhizal association when inoculated to leek plants under controlled conditions. The intraradical forms of *Glomus* sp. immobilized in alginate beads regenerated mycelium which colonized roots. (Strullu and Plenchette, 1992).

On colonization of roots VAM fungi improve the growth of the host plant mainly by the increased uptake of diffusion limited nutrients especially phosphorus. The principal function of mycorrhiza is to increase the soil volume explored for nutrient uptake and to enhance the efficiency of nutrient absorption especially P from the soil solution. (Rhodes and Gerdemann, 1975). Inoculation of cowpea with VAM fungus either as sand : soil inoculum or as alginate beads enhanced the biomass and phosphorus content in plant tissue (Table 9 - 11) when compared to that of uninoculated plants. In general there was not much difference in biomass and phosphorus content of plants inoculated with dry or wet VAM alginate beads. Biomass and P content of plants inoculated with sand : soil VAM fungal (*Glomus mosseae*) inoculum were almost on par with plants treated with alginate beads. Cowpea plants were inoculated with sand : soil VAM fungal inoculum and the different carrier based VAM wet or dry alginate containing 500 I.P. of *Glomus mosseae* each, hence there was not much difference in biomass and phosphorus content of cowpea. Bashan (1986) working with *Azospirillium* observed that there was no significant difference between inoculation with wet or dry alginate beads. The effect of inoculation of the VAM alginate beads on other crops and under field conditions requires further research. The quantity of beaded inocula added to obtain 500 I.P. per pot was least in dry and wet VAM beads having perlite as carrier material, as the propagule density was highest in perlite-alginate beads. Hence the dry perlite based VAM alginate beads would be the cheapest of the different carrier based alginate entrapped VAM inoculum (Table 12).

Formulation and application methods of microbiol inoculants are of paramount importance in agriculture. The process of immobilization in calcium alginate produces biodegradable pellets of uniform size. The size of the alginate beads and its composition can be varied (Fravel *et al.*, 1985; Bashan, 1986). Sodium alginate and calcium chloride are commonly used

Table 12 : Cost of producing 100 g of carrier based wet VAM alginate beads.

Requisites	Amount needed	Cost/kg (Rs)	Total cost (Rs) 100g	Cost of 100 g wet VAM beads (Rs)	Inoculation cost per pot (Rs)
1. Sodium alginate	2.7 g	560.00	1.512		
2. Distilled water	135.0 ml	-	-		
3. $CaCl_2$ solution					
a Distilled water	100.0 ml	-	-		
b. $CaCl_2$ pellets	2.2 g	90.00	1.198		
4 Sand:soil VAM inoculum	13.5 g	-	-		
Common cost			1.710		
5. Carrier materials					
a Perlite	2.7 g	13.65	0.036	1.746	2.30
b Soilrite	2.7 g	21.52	0.058	1.768	3.08
c Talc	2.7 g	30.00	0.081	1.791	4.11
d Vermiculite	2.7 g	10.00	0.020	1.737	6.95
e Kaolinite	2.7 g	162.00	0.437	2.147	11.30
f Bentonite	2.7 g	396.00	1.069	2.799	14.63

as food additives, hence are considered to be non-toxic (Fravel *et al.*, 1985). The alginate beads readily solubilize especially in the presence of calcium chelating ions, such as phosphate, citrate and EDTA (Brodelius and Vandamme, 1987). *Pseudomonas* sp. strain OS-ALG-9 which solubilized calcium alginate beads in a reactor was identified by Kinoshita *et al.* (1991). The enzyme alginate lyase which degrades alginate was produced both intra and extracellularly by the organism. The activity of the enzyme was optimum at pH 7 and temperature 30°C. Another group of scientists (Yonemotto *et al.*, 1991) isolated bacteria producing alginate lyase from ditch sample. The alginate lyase purified by them was most active at pH 8.0. Bashan (1986) reported that biodegradation of alginate beads in soil depends on soil microflora, its type and density. Degradation of beads was more complete in heavy texture soils than in sterile or light texture soils. Beads were completly degraded by 4 weeks in heavy texture soils, while in unsterile soils the beads began to degrade after 4 weeks perhaps as a result of soil contamination.

Inoculation technology employed for use of biofertilizers in fields should be simple and practical to the farmer, aiding its use and application with existing farm machinery. The dry alginate beads are convenient for storage and can be applied to soil easily, manually or with currently used agricultural machinery. Further more the alginate beads with biofertilizers or microbial inoculants entrapped in it can be applied simultaneously with seeds at the time of sowing. In rainfed farming the germinating seeds get infected by the microbial inoculant in the alginate beads as it rains. Slow release from the beads will ensure a constant supply of the microbial inoculant to the plant.(Bashan, 1986).

The alginate-perlite granules of *Rhizobium* are more suited for inoculation of crops with epigeal germination. This is because when the cotyledons emerge above the level as seed germinates most of the lignite or peat based rhizobial inoculum used for seed treatment also come out of the soil and hence will not be available for infecting the roots. In tropics and semi arid tropics where dryland farming is practiced re-inoculation of the microbial inoculant becomes a necessity because dry spells prevail. For rhizobial inoculations under such conditions, it was pointed out that alginate - perlite beads could possibly overcome the need for re-inoculation (Hegde and Brahmaprakash, 1992). Xanthan alginate entrapped rhizobial (XER) beads inoculation to plants was comparable in nodulation and growth parameters to that of peat based rhizobial inoculation. But the XER beads inoculation could possibly overcome the complication of damping off sometimes associated with the application of peat based inoculants (Jung *et al.*, 1982).

The loose sand : soil inoculum of VAM fungi is known to clog the pipes of farm machinery but not the granulated inoculum during field application (Powell and Bagyaraj, 1982). The dry VAM alginate beads, which is granular and having several advantages mentioned earlier could be conveniently used in farm machinery . The VAM fungi has synergistic interactions with several beneficial rhizosphere microorganisms, like legume bacteria (Manjunath and Bagyaraj, 1984), free living nitrogen fixing, phosphate solubilizing bacteria (Manjunath *et al.*, 1981) and beneficial actinomycetes (Krishna *et al.*, 1982). Mycorrhizae helper bacteria (MHB) have recently been reported to enhance colonization by VAM fungi with consequential benefit on plant growth (Machado and Bagyaraj, 1995) Thus an inoculum of VAM fungus and the beneficial soil microorganism in calcium alginate beads could be introduced to soil by a single application using the entrapment technology. This aspect of introducing one or more beneficial rhizosphere bacteria along with VAM fungi to soil using alginate beads by a single application needs further investigation.

The production of alginate entrapped VAM inoculum is costlier when compared to that of sand : soil inoculum. A high propagule density of VAM fungi could be obtained by concentrating the sand : soil inoculum by sieving (Hayman *et al.*, 1981) or also by producing VAM fungal infected roots by aeroponics (Hung and Sylvia, 1988) or hydroponics (Mosse and Thompson, 1984) or root organ culture (Becard and Piche, 1992), viable spore isolation by wet sieving and decantation or sucrose centrifugation. These roots and spores of VAM fungi entrapped in calcium alginate would regenerate and infect plant roots on inoculation. The drawbacks of the VAM root inoculum is its short viability on storage. If the root inoculum could be mascerated and entrapped in alginate beads, its shelf life can definitely be increased further. Hence the concentrated VAM inoculum having high propagule density when immobilized in calcium alginate beads the amount to be inoculated to soil, the transportation and storage charges could be drastically reduced. Bashan (1986) emphasizes that the alginate beads fulfill many of the requirements for a good practical inoculant. The alginate beads could be dried, they are synthetic, simple to use, uniform in size its composition being versatile, biodegradable by soil micro organisms, non toxic in nature, provides slow release of the microbial inoculant and causes no ecological pollution.

SUMMARY

VI. SUMMARY

An investigation was conducted to know the possibility of producing alginate entrapped VA mycorrhizal inoculum. The inoculum potential and the survival of VAM propagules in the alginate beads was also determined. Furthermore the effect of inoculation of the VAM alginate beads on growth and P uptake of cowpea was also studied.

Perlite, soilrite, talc, vermiculite, kaolinite and bentonite were the different carrier materials immobilized along with sand : soil inoculum of *Glomus mosseae*, to prepare substrate based VAM alginate beads. A concoction containing sodium alginate (2%), carrier material (2%) and sand : soil inoculum of *Glomus mosseae* (10%) were mixed thoroughly and added dropwise to sterile distilled 0.1 M calcium chloride solution to obtain VAM alginate beads.

Infective propagule (I.P.) numbers of *Glomus mosseae* in alginate entrapped VAM beads was determined by the MPN method using four fold dilutions. The MPN method gave a relative measure of the inoculum potential of the different substrate based beaded inocula. Perlite based wet VAM alginate beads had a maximum of 3.79 I.P. per g of the inocula followed by soilrite (2.87 I.P/g), talc (2.18 I.P./g), vermiculite (1.25 I.P./g), bentonite (0.95 I.P./g) and kaolinite (0.95 I.P./g). The I.P. numbers of dry VAM beads were derived from the I.P. numbers of wet VAM beads and its moisture percent; hence the MPN value of dry VAM beads were higher and the same trend of the inoculum potential of the different carrier based wet VAM beads was also seen in the dry VAM alginate beads.

The wet carrier based beads were stored at 5°C in a refrigerator, the dry beads were packed in polythene covers and stored in at room temperature (30 ± 5°C). The VAM propagules in alginate beads were viable for 6 months (maximum period tested in this study) and could colonize onion roots. A decline in the percent root colonization was noticed as the days of storage increased. This decline was more pronounced in dry alginate beads compared to wet beads.

Out of the 6 carrier based VAM alginate beads, only 4 beaded inocula which supported high propagule numbers of VAM fungi were used to study plant growth response. Cowpea was inoculated with sand : soil based, wet and dry alginate entrapped inoculum prepared using perlite, soilrite, talc and vermiculite as carrier materials. Sand : soil inoculum of *Glomus mosseae* was used as standard. This experiment contained 10 treatments, including a uninoculated control. Soil in each pot was mixed well with the required amount of inocula, so that each pot received 500 I.P. of the VAM fungus. The alginate entrapped VAM beads colonized roots of cowpea and the percent colonization varied from 67.99 to 70.91. The extent of VAM root colonization was highest in plants which received sand : soil inoculum (76.25%). Inoculation of cowpea with VAM fungi either as sand : soil inoculum or as alginate entrapped inoculum (wet or dry) enhanced the biomass and phosphorus content of cowpea. In general there was not much difference in the biomass and phosphorus content of plants inoculated with sand : soil VAM inoculum and wet or dry alginate entrapped VAM inoculum.

Formulation of VAM inoculum is of importance, as it aids easier application and use of the inoculum at field conditions. The dry VAM alginate beads though costlier than sand : soil inoculum has many advantages.

These VAM beads are easy to prepare, simple to use, biodegradable and its composition can be varied. The beaded inoculum provides slow and constant release of the inoculum to plants. It is non-toxic in nature and causes no ecological problem. The alginate beads could be used for the introduction of VAM fungi along with other beneficial soil microorganisms which promote plant growth.

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VII. REFERENCES

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*Original not seen

APPENDICES

APPENDIX - I

RUAKURA PLANT NUTRIENT SOLUTION

1. Major elements

		g/4.5 litres
Solution A	$Mg(NO_3)_2.6\ H_2O$	22.25
	$Ca(NO_3)_2.4H_2O$	75.50
	NH_4NO_3	38.40
	KNO_3	10.25
Solution B	NaCl	1.50
	$^*KH_2PO_4$	12.00
	$^*K_2HPO_4$	7.40
	K_2SO_4	29.783
	NA_2SO_4	2.691

2. Minor elements

		g/500 ml	
a) Boron	H_3BO_3	6.3831	Dissolve in 500 ml water
b) Manganese	$MnCl_2.4H_2O$	20.2647	Dissolve in 20.26 ml of 0.1N HCl make up to 500 ml with water.
c) Zinc	$ZnCl_2$	5.864	Dissolve in 117.3 ml of 0.1 N HCl and make upto 500 ml with water.
d) Copper	$CuCl_2.2H_2O$	1.2073	Dissolve in 12.07 ml of 0.1 N HCl and make upto 500 ml with water.
e) Molybdenum	$(NH_3)_6Mo_7O_{24}.4H_2O$	0.2070	Dissolve in 500 ml water
d) Cobalt	$CoCl_2.H_2O$	0.4088	Dissolve in 500 ml water

Place 5 ml of each of a, b, c, d, e and f in a bottle and dilute to 2.5 litres with water.

3. Iron :

Dissolve 13.5 g Ferric citrate ($FeC_6H_5O_7.H_2O$) in 119 ml of IN HCl and dilute to 2.5 litres with water.

To Make up the solution :

Add 300 ml A, 300 ml of B, 150 ml of 2 (diluted) and 22.5 ml of Ferric citrate and make up to 4.5 litres with water.

(Omit K_2HPO_4 and KH_2PO_4 for nutrient solution minus phosphorus)

APPENDIX II

Vanadomolybdate reagent

Solution A is prepared by dissolving 25 g ammonium molybdate in 400 ml of distilled water. Solution B is prepared by dissolving 1.25 g of ammonium metavanadate in 300 ml boiling water. Solution B is cooled and then 200 ml of concentrated HNO_3 is added and the solution is again cooled to room temperature. Finally solution A is poured into solution B and the mixture is diluted to one litre in a volumetric flask.