DEVELOPMENT AND EVALUATION OF ALGINATE BASED MICROBIAL CONSORTIA OF AGRICULTURALLY BENEFICIAL MICROORGANISMS

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CERTIFICATE

This is to certify that thesis entitled **"DEVELOPMENT AND EVALUATION OF ALGINATE BASED MICROBIAL CONSORTIA OF AGRICULTURALLY BENEFICIAL MICROORGANISMS"** submitted in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in Agricultural Microbiology to the University of Agricultural Sciences, Bangalore, is a bonafide record of research work carried out by **Mr. SUBRAMANYAM, B.** during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis of the award of any other degree, diploma, associateship, fellowship or similar other titles.

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DEVELOPMENT AND EVALUATION OF ALGINATE BASED MICORBIAL CONSORTIA OF AGRICULTURALLY BENEFICIAL MICROORGANISMS

SUBRAMANYAM, B.

ABSTRACT

Experiments were conducted to develop microbial consortia of agriculturally beneficial microorganisms (ABMs) and to determine their effectiveness on plant growth in greenhouse conditions. Laboratory experiment was conducted to check alginate bead formation with different plant nutrient cations such as Mg, Mn, Zn and Ca. Results revealed that beads are smooth and stable with calcium cations and hence Ca-alginate beads were selected. Further experimentation to optimize concentrations revealed that 2 M sodium alginate with 0.2 M $CaCl_2$ formed appropriate beads for consortia. Isolates of *Azotobacter* sp., *Acinetobacter* sp. and *Pseudomonas* sp. obtained from the department of agricultural microbiology, UAS, GKVK, were used for preparation of consortia in this study. Consortia were developed by entrapping the Cells of bacterial inoculants into calcium-alginate beads. Population densities in these beads were determined soon after their preparation. Beads were further stored for ten months in refrigerated and in ambient temperatures and population density in those stored beads was also determined. Results indicated a population density of more than 10^7CFUs of bacteria per gram of beads, as recommended by Bureau of Indian Standards (BIS), even after 300 days of storage. Greenhouse experiments conducted to evaluate the effectiveness of consortia on plant growth and nutrition revealed a significant improvement in plant nutrition and biomass for inoculation of soil with consortium of ABMs. Further, determination of optimal soil conditions, fertilization levels, microbial inoculants of consortia that benefit plants at most, would assure a more sustained growth response of plants for inoculation to consortia of ABMs.

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(Major Advisor)

ಕೃಷಿ ಪ್ರಮುಖವಾದ ಸೂಕ್ಷ್ಮಜೀವಿಗಳ ಒಕ್ಕೂಟದ ಸೂತ್ರಿಕರಣಗಳ ಅಭಿವೃದ್ಧಿ ಮತ್ತು ಗಿಡದ ಬೆಳವಣಿಗೆಯ ಮೇಲೆ ಅವುಗಳ ಪ್ರಭಾವ

ಸುಬ್ರಮಣ್ಯಂ, ಬಿ.

ಪ್ರಬಂಧ ಸಾರಾಂಶ

ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ ಕೃಷಿ ಪ್ರಮುಖವಾದ ಸೂಕ್ಷ್ಮಜೀವಿಗಳ ಒಕ್ಕೂಟದ ಸೂತ್ರಿಕರಣಗಳ ಅಭಿವೃದ್ಧಿ ಹಾಗೂ ಅವುಗಳ ಪ್ರಭಾವ ಗಿಡದ ಬೆಳವಣಿಗೆಯ ಮೇಲೆ ಏನಾಗುವುದೆಂದು ತಿಳಿಯಲು ಸಂಶೋಧನೆಗಳನ್ನು ಕೈಗೊಳ್ಳಲಾಯಿತು. ಪ್ರಯೋಗಾಲಯದಲ್ಲಿ ಆಲ್ಜಿನೇಟ್ ಜೊತೆ ವಿವಿಧ ಸಸ್ಯ ಪೋಷಕಾಂಶಗಳಾದ ಮೆಗ್ನೀಶಿಯಂ, ಮ್ಯಾಂಗನೀಸ್, ಜಿಂಕ್ ಮತ್ತು ಕ್ಯಾಲ್ಸಿಯಂ ಲವಣಗಳನ್ನು ಬೆರೆಸಿ, ಅವುಗಳ ಹರಳುಗಳು ಆಗುವದನ್ನು ಅಧ್ಯಯನ ಮಾಡಲಾಯಿತು. ಕ್ಯಾಲ್ಸಿಯಂ–ಆಲ್ಜಿನೇಟೆ ಹರಳುಗಳನ್ನು ಉಳಿದೆಲ್ಲವುಗಳಿಗಿಂತ ಈ ಆಧ್ಯಯನಕ್ಕೆ ಸೂಕ್ತವೆಂದು ಆರಿಸಿಕೊಳ್ಳಲಾಯಿತು. ಪ್ರಯೋಗದಲ್ಲಿ ಉಪಯೋಗಿಸಲಾದ ವಿವಿಧ ಪ್ರಮಾಣಗಳಲ್ಲಿ, 2 ಮೋಲಾರ್ ಸೋಡಿಯಮ್ ಆಲ್ಜಿನೇಟ್ ಮತ್ತು 0.2 ಮೋಲಾರ್ ಕ್ಯಾಲ್ಸಿಯಂ ಕ್ಲೋರೈಡ್ ಅನ್ನು ಸಂಯುಕ್ತ ಸೂಕ್ಷ್ಮಜೀವಿಗಳ ಹರಳುಗಳನ್ನು ತಯಾರಿಸಿಲು ಸೂಕ್ತವೆಂದು ಪರಿಗಣಿಸಲಾಗಿದೆ. ಆಯ್ದ ಸೂಕ್ಷ್ಮ ಜೀವಿ ಪ್ರಬೇಧಗಳಾದ ಅಝಟೋಬ್ಯಾಕ್ಟರ್, ಅಸಿನೆಟೋಬ್ಯಾಕ್ಟರ್ ಮತ್ತು ಸೂಡೋಮೋನಾಸ್ ಗಳನ್ನು ಕೃಷಿ ಸೂಕ್ಷ್ಮಾಣು ಜೀವಿ ವಿಭಾಗ, ಕೃಷಿ ವಿಶ್ವ ವಿದ್ಯಾನಿಲಯ, ಜಿ.ಕೆ.ವಿ.ಕೆ, ಇಲ್ಲಿಂದ ಪಡೆಯಲಾಗಿದೆ. ಆಯ್ದ ಸೂಕ್ಷ್ಮಾಣುಗಳನ್ನೊಳಗೊಂಡ ಉಪಯೋಗಿಸಿಕೊಂಡು ಕ್ಯಾಲ್ಸಿಯಂ–ಆಲ್ಜಿನೇಟೆ ಹರಳುಗಳನ್ನು ತಯಾರಿಸಲಾಯಿತು. ಇವುಗಳನ್ನು ಹತ್ತು ತಿಂಗಳುಗಳವರೆಗೆ ಶೀತ ಹಾಗೂ ಸಾಮಾನ್ಯ ತಾಪಮಾನ ಗಳಲ್ಲಿ ಶೇಕರಿಸಿ ಇಡಲಾಯಿತು. ಈ ಹರಳುಗಳಲ್ಲಿ ಇರುವ ಸೂಕ್ಷ್ಮ ಜೀವಿಗಳ ಪ್ರಮಾಣವನ್ನು ಅವುಗಳನ್ನು ತಯಾರಿಸಿದ ಬಳಿಕ ಹಾಗೂ ಹತ್ತು ತಿಂಗಳುಗಳ ಕಾಲ ಶೀಕರಿಸಿದ ನಂತರ ನಿರ್ಧರಿಸಲಾಯಿತು. ಪರಿಶ್ಕೃತ ನಿಗಧಿಪಡಿಸಲಾಗಿರುವ ಪ್ರಮಾಣಕ್ಕಿಂತ ಹೆಚ್ಚಿನ ಪ್ರಮಾಣದಲ್ಲಿ ಆಯ್ದ ಸೂಕ್ಷ್ಮ ಜೀವಿಗಳ ಇರುವಿಕೆಯನ್ನು ಇದರಿಂದ ಖಾತರಿ ಪಡೆದುಕೊಳ್ಳಲಾಯಿತು. ಆಯ್ದ ಸೂಕ್ಷ್ಮ ಜೀವಿಗಳನ್ನೊಳಗೊಂಡ ಹರಳುಗಳ ಉಪಯೋಗದಿಂದ ಗಿಡಗಳ ಬೆಳವಣಿಗೆಯ ಮೇಲೆ ಆಗುವ ಪರಿಣಾಮವನ್ನು ಹಸಿರು ಗಾಜಿನಲ್ಲಿ ಪರೀಕ್ಷಸಲಾಯಿತು. ಈ ಅಧ್ಯಯನಗಳಿಂದ ದೊರೆತ ಉತ್ತರಗಳ ಪ್ರಕಾರ, ಕೃಷಿ ಪ್ರಮುಖವಾದ ಸೂಕ್ಷ್ಮಜೀವಿಗಳ ಒಕ್ಕೂಟದ ಸೂತ್ರಿಕರಣಗಳ ಬಳಕೆಯಿಂದ ಗಿಡಗಳ ಬೆಳವಣಿಗೆಯು ಗಣನೀಯವಾಗಿ ಪ್ರೋತ್ಸಾಹಿಸಲ್ಪಡುತ್ತದೆ ಎಂದು ತಿಳಿದುಬಂದಿದೆ.

ಸೆಪ್ಟೆಂಬರ್, ೨೦೧೫

ಕೃಷಿ ಸೂಕ್ಷ್ಮಜೀವಿಶಾಸ್ತ್ರ ವಿಭಾಗ
ಕೃಷಿವಿಶ್ವವಿದ್ಯಾನಿಲಯ
ಗಾಂಧಿ ಕೃಷಿ ವಿಜ್ಞಾನ ಕೇಂದ್ರ, ಬೆಂಗಳೂರು

ಎ. ಮಂಜುನಾಥ್
(ಮುಖ್ಯ ಸಲಹೆಗಾರರು)

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INTRODUCTION

I. INTRODUCTION

Soil microorganisms influencing plant growth has been noticed since time immemorial. Numerous mechanisms through which microorganisms help in plant growth have been investigated. Also, the microorganisms involved have been categorized. Symbiotic nitrogen fixers are the category of microorganisms that benefit a group of plants most by nitrogen nutrition followed by asymbiotic nitrogen fixers, P solubilizers and mobilizers, and plant growth promoting rhizobacteria (PGPR). All these microbes are collectively referred to as agriculturally beneficial microorganisms (ABMs). Formulations have been developed with selected ABMs to harness the capabilities of these microbial entities for benefitting agricultural crops. Accordingly, application of these formulations also have shown considerable success. However, application of these formulations at field level is far less than the extent at which organic and chemical fertilizers are accepted and used. Limited acceptance is mainly due to the inconsistent crop response to these bioinoculant formulations and the major root cause for ineffective formulations is due to poor survival and establishment rate of bioinoculants in the introduced ecosystems.

Soil zone influencing itself as well as being under the influence of plant root system is referred to as rhizosphere. This has significantly higher native microbial activity than non rhizosphere soil. The microbial activity in rhizosphere could be beneficial, harmful or neutral to the plants (Dommergues, 1978). For the introduced microbial strains to have a better chance to survive and succeed in this zone of competition and struggle, they have to be introduced in sufficiently high numbers and need to be protected and nurtured to their best. Further studies in natural ecosystems have revealed that that the complex groups of microbes survive better by withstanding different stress conditions than being independent.

Thus consortial formulations of ABMs are presumed to serve as effective bio inoculants in field conditions. Alagawadi and Gaur (1988) and many other workers have observed that the combined inoculation of two or three beneficial microorganisms perform better than single strain inoculations. Significantly higher response of plants have been commonly noticed in case of dual inoculation of nitrogen fixing and phosphate solubilizing bacteria and in the case of mycorrhizal fungi and mycorrhizal helper bacteria (Kundu and Gaur, 1984 and Gamalero *et al.*

2004). Triple inoculation of a nitrogen fixer, phosphate solubilizer and a mycorrhizal fungus had shown significant increase in plant growth and yield (Belimov *et al.*1995 and Khan and Zaidi, 2007). A formulation containing more than one microorganism doesn't exist despite the recorded benefits of dual and triple inoculations. With these observations, formulations of consortia that include not one but a few ABMs in single formulation are hypothesized to be an alternative to the limitations posed by single inoculant formulations. Consortial formulations are said to be ideal if they don't allow any negative interactions among the resident microorganisms (Smith, 1992).

Digat (1993) developed a new encapsulation technique that results in the formation of macrocapsule consisting of a core and an envelope. This system offers the possibility of encapsulating simultaneously two types of microorganisms one in the core and another in the envelope. He proposed that mixed microbial cultures in the encapsulated capsule allow their components to interact with each other synergistically. In continuation with this hypothesis, Veena (1999) and Bashan (1998) demonstrated that consortia of soil microbial cultures with similar but different functional capabilities could be developed and introduced into rhizosphere. Vassilev *et al.* (2001) formulated a capsule consisting of a nitrogen fixing bacterium and a phosphate solubilizing yeast. Further, the importance of microbial interactions in mycorrhizosphere for improving plant growth and soil health has been reviewed by Barea *et al.,* (2002). Although attempts have been made to develop formulations of ABMs, effect of their inoculation under defined soil conditions has not been demonstrated.

From the review of existing carrier materials and formulation techniques, alginate bead formulations are considered to be the best to develop consortial formulations. Entrapment and encapsulation of microorganisms in alginate beads protects them from environmental stress conditions and release microorganisms gradually as and when alginate beads get degraded and thus may serve as continuous source of inoculum in rhizosphere. Storage of these beads at ambient temperature has been found to retain the inoculum in viable condition for a prolonged period of more than a year. Also, no harmful effect of alginate polymer on plant growth has been noticed.

In this context, the present work has been taken up with the following objectives

1. To develop alginate based microbial consortia of agriculturally beneficial microorganisms (ABM).
2. To evaluate effectiveness of microbial consortia under green house conditions using selected indicator plant species.
3. To determine the survivability of ABM in consortia under defined storage conditions.
4. To monitor the effectiveness of vesicular arbuscular mycorrhizal (VAM) fungus in microbial consortia using selected indicator plant.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Microbial consortia refers to multiple interacting microbial populations. Most microbes live in heterogeneous groups of surface bound congregations called biofilms. In many cases microbes in biofilms develop into complex interactive communities called consortia. Members of the consortium (consors) communicate with one another which enable them for the division of labor. The overall output of a consortium rests on combinations of tasks performed by its sub populations.

Consortia can perform functions that are difficult or even impossible for individual strains or species and they are robust to environmental fluctuations. This has made synthetic biologists to hone their ability to engineer microbial consortia which are far superior to their constituent mono populations (Brenner *et al*., 2008).

The concept of using biofilmed biofertilizers (BBs) is considered to be a novel biofertilizing technique. This involves culturing agriculturally beneficial micro organisms (ABMs) together to form biofilms and they are introduced to soil as biofertilizers (Seneviratne and Jayasinghearachchi, 2005). Recent studies demonstrated that biofilms grown in axenic conditions using one, two or more bacterial species with fungus have better activity than their individual populations (Santaella *et al.,* 2008 and Seneviratne, *et al.,* 2009). Their studies reveal that BBs establish an association with the plant roots and addition of chemical nutrients increases the microbial biomass of biofilms.

Biofilms containing nitrogen fixers and other organisms act as pseudonodules which fix nitrogen and release organic acids (Mongiardini *et al.,* 2008). The acidic conditions may suppress microbial pathogens as well as help in mineralization of soil nutrients in the rhizosphere. Also plant growth hormones such as IAA produced by the biofilms increase growth of roots and mycorrhizal fungi. Thus microbial associations in biofilms constitute an excellent metabolic cooperation for healthy growth of the plant and are known to supplement fifty per cent of chemical fertilizer requirement of the tested plants.

However, selection of combination of microbes possessing the highest efficiency with respect to plant growth and soil nutrient management has to be considered in the preparation and formulation of consortia.

2.1 Benefits of microbial associations in agriculture

Soil microorganisms have been differentiated by soil microbiologists and microbial ecologists as beneficial and harmful according to their functions. Beneficial microorganisms are those that can stimulate plant growth by various mechanisms such as fixing atmospheric nitrogen, decomposing organic residues and enhancing nutrient cycling, detoxifying pesticides, suppressing plant diseases, soil borne pathogens and by producing bioactive compounds such as vitamins, hormones, enzymes etc. Using some of these beneficial microorganisms, various microbial inoculants have been prepared for use in crop production to reduce the cost on chemical fertilizers and to minimize environmental pollution.

Since microbial inoculants are useful in eliminating the problems associated with use of chemical fertilizers and pesticides, they are now widely applied in nature farming and organic agriculture (Higa, 1991). These inoculants have although met with considerable success, they have not been widely accepted because of inconsistent performance under field conditions.

Combined use of two or three beneficial microorganisms as inoculants have been found to perform better than single inoculations (Alagawadi and Gaur, 1988). Plant growth enhancement due to the inoculation of either single strain or combination of microbial strains has been noticed in several investigations and the mechanisms of growth improvement is attributed mainly to nitrogen and phosphorus nutrition as well as production of growth hormones and suppression of plant pathogens.

A formulation containing more than one microorganism does not exist despite recorded benefits of dual inoculations. A single formulation containing consortium of agriculturally beneficial microorganisms needs to be developed as they play crucial role in enhancing productivity and maintaining soil health. An ideal microbial consortium formulation is the one that does not allow any negative interactions among the resident microorganisms (Smith, 1992). The negative interactions in a formulation may decrease number of viable cells, affecting the effectiveness of consortium, which emphasizes the need for the development of ideal formulation which accelerate and improve plant growth and protect plants from pests and diseases (El-yazeid *et al.,* 2007).

2.2 Beneficial interactions of microorganisms and plants

Hiltner (1904) designated the term rhizosphere for the region of soil where he noticed interaction between plant roots of legume and that of soil bacteria. The rhizosphere microflora differs qualitatively and quantitatively from that of non-rhizosphere soil, their influence on plant growth is also significantly different (Rangaswami and Vasantharajan, 1962 and Bowen and Rovira, 1976).

High microbial activity in rhizosphere is because of the extra nutrients available from root exudates, sloughed off root tissues, mucigels *etc.,* (Curl and Truelove,1986; Darrah,1991; Whipps and Lynch,1985 and Dhruvakumar, *et al., 1992*).Microorganisms of different taxonomic and nutritional groups found in rhizosphere have been classified as being harmful, neutral and beneficial to the plants (Dommergues, 1978). Biofertilizers are products containing living cells of different types of microorganisms which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus) from unavailable to available form through biological process such as nitrogen fixation and solubilization of rock phosphate (Rokhzadi *et al .,* 2008).

2.3 Agriculturally beneficial microorganisms (ABMs)

Soil harbors a vast array of bacteria, actinomycetes, fungi and algae which support plant growth and in general they are referred to as ABMs. These diverse functional groups of microorganisms are broadly categorized as nitrogen fixers, phosphate solubilizers, plant growth promoting rhizobacteria and vesicular Arbuscular mycorrhizal (VAM) fungi.

The most common symbiotic nitrogen fixers are *Rhizobium* and *Bradyrhizobium* that are capable of forming symbiotic association with legumes. The common free living nitrogen fixers are *Azotobacter* and *Azospirillum.* These organisms fix atmospheric nitrogen and supplement for the nitrogen source of plants.

Several bacterial species are capable of increasing availability of phosphorous to plants by solubilizing organic or inorganic phosphates by producing organic acids (Rodriguez and Fraga, 1999). These bacteria are referred to as phosphate solubilizing bacteria (PSB) and have been considered to have potential use as bio inoculants to improve plant growth and yield (Yadav and Dadarwal, 1997, Rodriguez and Fraga, 1999, Vessey 2003, Chen *et al*., 2006). PSBs are common for all crops and several species of them occur in soil but usually their numbers are not enough to compete with native bacteria.

Plant growth promoting rhizobacteria (PGPR) are the root colonizing bacteria (rhizo bacteria) that exert beneficial effects on plant development either by direct or indirect mechanisms. Direct mechanism of plant growth improvement is by producing plant growth promoting substances (Gaskins *et al*., 1985). Indirect plant growth promotion occurs when bacteria offsets the deleterious effects of phyto-pathogenic organisms (Zehnder *et al.,* 2000).*Pseudomonas* sp. are at present receiving worldwide attention as PGPRs (Kloepper *et al.,* 1989).

Arbuscular mycorrhizal fungi are one of the predominant soil organisms that colonize root system of most of the terrestrial plants and promote plant growth mainly by enhanced phosphorus absorption (Gerdemann, 1968 and Bolan, 1991).

Three major groups of microorganisms are considered beneficial to plant nutrition, arbuscular mycorrhizal fungi (AMF) (Jeffries *et al.,* 2003), plant growth promoting rhizobacteria (PGPR) (Podile and Kishore, 2006), and nitrogen-fixing rhizobia, which are usually not regarded as PGPR(Franche *et* al., 2009).

2.4 Plant nutrition from selected ABMs

2.4.1 Nitrogen fixing microorganisms

Nitrogen is the primary nutrient requirement of the plant. Microorganisms that have the capability to fix atmospheric nitrogen and make it available to the plants have been observed and utilized to improve plant growth. Apart from *Rhizobium* sp. which are symbiotically associated with legume plants and fix atmospheric nitrogen in significant quantities, associative and free living nitrogen fixers have been noticed and are equally important to harness atmospheric nitrogen for non leguminous plants and soil improvement.

Azotobacter chroococcum on an average has recorded 10 mg of nitrogen per gram of carbon source utilized (Thomas, 1993). Joshi *et al*. (2007) recorded up to 13 mg of nitrogen fixation per gram of carbon source used. Inoculation of *Azotobacter* sp. has resulted in higher concentrations of nitrogen in plant tissues and increase in yield parameters of crop plants (De Freitas, 2000; Kumar *et al.*, 2001 and Konde and Shinde, 1986). *Azotobacter* sp. along with *Azospirillum* sp. have shown a positive improvement in plant growth parameters.

Inoculation of *Azotobacter chroococcum* has shown significant improvement in plant growth and yield and has demonstrated savings on nitrogenous fertilizer in significant quantities (Anjum *et al.,* 2007 and Ananthanaik *et al.,*2007). Use of *Azotobacter* has been recommended as an alternative to chemical fertilizers (Rajeshwari *et al*., 2007) to minimize the use of harmful substances in agriculture. Thus *Azotobacter chroococcum* has proved to be a promising free living nitrogen fixer and could serve to replenish nitrogen content in soil.

Biological nitrogen fixation is one way of converting elemental nitrogen into plant usable form (Gothwal *et al*., 2007). Nitrogen-fixing bacteria (NFB) that function transform inert atmospheric N2 to organic compounds (Bakulin *et al* ., 2007). Nitrogen fixer or N-fixers organism are used in biofertilizer as a living fertilizer composed of microbial inoculants or groups of microorganisms which are able to fix tmospheric nitrogen. They are grouped into free-living bacteria (Azotobacter and Azospirillium) and the blue green algae and symbionts such as Rhizobium, Frankia and Azolla (Gupta, 2004).

2.4.2 Phosphate solubilizing bacteria and P-mobilizers

Phosphorus in soil is present in the form of insoluble precipitates and is unavailable for plant uptake. Certain microorganisms have been identified to aid in phosphorus plant nutrition by different mechanisms. A significant correlation between the number of phosphate solubilizing microorganisms and that of the total available phosphorus in soil was noticed (Kucey, 1983). Many bacteria and fungi have been identified with different mechanisms that aid in phosphorus nutrition for the plants.

Vesicular Arbuscular Mycorrhiza (VAM) refer to the symbiosis of soil fungus with plant roots that mainly benefit plants by mobilizing phosphorus. Infection with mycorrhizal fungi improves phosphorus nutrition of host plants growing in poor soils (Smith and Gianinazzi-Pearson, 1998; Bolan, 1991). The role played by them in tropics is of prime agricultural importance where availability of phosphorus is a limiting factor for plant productivity (Sanchez and Uehera, 1980; Bethlenfalvay, 1992 and Habte and Osorio, 2001).

Among bacteria, *Bacillus* sp. has been considered as an efficient Phosphate Solubilizing Bacteria (PSB) and their formulations have been developed and used. *Acinetobacter* sp. is also an efficient phosphate solubilizing bacteria (Fan *et al.,* 2011). In addition to phosphate solubilization, *Acinetobacter* sp. is also recorded to be producing different extracellular gibberellins and thus promoting plant growth (Kang *et al.*, 2009).

Hilda and Fraga (1999) described *Acinetobacter* sp. as a PGPR apart from being a phosphate solubilizer. Production of organic acids by *Acinetobacter* sp. and concomitant increase in plant growth by enhanced phosphorus nutrition has been evidenced by Gulati *et al.,* (2010). Thus formulations delivering *Acinetobacter* sp. could promise increased phosphate solubilization and improvement in plant growth by enhanced phosphorus nutrition.

A selected strain of *Acinetobacter* sp. studied for their plant growth promotion mechanisms have shown plant growth-promoting attributes of inorganic and organic phosphate solubilization, auxin production, ammonia generation and siderophore production. The study has also demonstrated the significant increase in growth of pea, chickpea, maize, and barley due to inoculation by selected *Acinetobacter* sp. under controlled conditions and in field conditions pea has showed a significant increment in plant growth and yield (Gulati *et al*., 2009). The phospho-microorganism mainly bacteria and fungi make insoluble phosphorus available to the plants (Gupta, 2004). Several soil bacteria and a few species of fungi possess the ability to bring insoluble phosphate in soil into soluble forms by secreting organic acids (Gupta, 2004). These acids lower the soil pH and bring about the dissolution of bound forms of phosphate.

2.4.3 Plant growth promoting rhizobacteria (PGPR)

Pseudomonas fluorescens have shown nitrogen fixation, iron and phosphate mobilization activities (Gupta, 1995). Inoculation of *Pseudomonas* sp. controlled the disease incidence, improved plant growth and yield (Haggag and Saber, 2000). Bio control activity of *Pseudomonas* was evidenced by Moenne-Loccoz *et al*., (1999). Co-inoculation of two strains of *Pseudomonas* sp. have also showed improved plant growth and yield (Sarma *et al*., 2009).

Inoculation of *Pseudomonas* sp. along with *Rhizobium* sp. significantly improved the plant biomass and nodulation of plant than their individual inoculations, thus ensuring positive interaction effect of *Pseudomonas* sp. with other ABMs (Arora *et al.,* 2008).Significant improvement in yield of commercial rice cultivars has been documented by *Pseudomonas* sp. (Mirza *et al.,* 2006). Significant improvement in plant growth and yield of tomato plants due to inoculation with *Pesudomonas* sp. has also been documented (Minorsky, 2008).

Simultaneous inoculation with different PGPR and/or AMF often resulted in increased growth and yield, compared to single inoculation through improved nutrient uptake (Belimov *et al.*, 1995 and Barea *et el.*, 2002). Indeed, the interactions between bacteria and AM fungi have beneficial functions related to nutrient uptake, particularly with PGPR (Barea *et el.,* 2002 and Vassilev *et al.,* 2001).

2.5 Formulations of ABMs

The ideal carrier will have to be non toxic to the microorganism that serves as inoculant and seeds on which they act. Further high organic matter content, high water holding capacity and high surface area are preferred characteristics of the carrier material. Essential pre requisites for large scale promotion of ABMs are development of viable, cost-effective and user-friendly formulations that are easy to handle and convenient for application. The ability of a formulation to consistently deliver critical number of viable cells is considered to be an indicator of its success (Pauu, 1988). Since the active ingredients of bio inoculant formulation is live microbial cells or spores, maintenance of inoculum in a metabolically and physiologically competent state is important in order to derive maximum advantage of the formulation. Other components of formulation, carrier and additives support the bacterial growth and multiplication nand are known to stabilize and protect inoculants during storage, transport and at the target zone. Further, for the same organism different formulations could be essential to suit different climates, soil types and end user preferences.

Researchers have attempted to develop formulations of ABMs with garden soil (Madhok, 1934), peat (Iswaran *et al*., 1969), cellulose powder (Pugashetti *et al*., 1971), coffee husk and forest soil, coir dust (Iswaran, 1972), rice husk and sand (Khatri *et al*., 1973), peat and bagasse (Graham *et al*., 1974), coal (Dube *et al*. 1975), sugarcane press mud and coffee waste (Kumar Rao *et al*., 1982), charcoal and vermiculite (Sparrow and Ham, 1983) as carrier material. Kandasamy and Prasad (1971) reported lignite carrier material as a good substitute for peat.

Though peat is the most popular carrier for bio inoculant formulations worldwide, the problem of unavailability of good quality peat is the major constraint. As far as India is concerned lignite and coal are considered to be the better choice as carrier material. But, the formation of hard clumps upon drying and during storage period is an associated problem which reduces the inoculant population in these formulations. Alginate based formulations are being evaluated and seem to offer substantial practical advantage over peat. These formulations are known to encapsulate living cells, which protect them against stress factors of soil and release them to rhizosphere gradually by slow degradation of alginate.

Coal, clays, and inorganic soils are available in different regions and can be used as carriers (Smith, 1995). Their microbial load depends on the site of production but it is generally lower than inorganic carriers. Vermiculite, perlite and bentonite are also available in different countries, but their use is generally limited due to the difficulty in creating a formulation (Millner and kitt, 1992).

2.6 Inoculation effect of ABMs on plant growth

Inoculation effect of diverse functional groups of ABMs on plant growth has been investigated by researchers. ABMs might improve plant growth by increased plant nutrition and also by stimulating plant hormone production (Bashan and Holguin, 1997).

Enhanced growth, biomass and nitrogen content of plants for inoculation of soil with nitrogen fixers have been observed (Ananthanaik *et al.,* 2007). Inoculation with *Acinetobacter,* which acts as P solubilizer and PGPR resulted in increased root length, seedling vigor and dry biomass of the canola plants and biomass of tomato (Indiragandhi *et al*., 2008). Field testing of pea with *Acinetobacter* has also showed significant increment in plant growth and yield (Gulati *et al*., 2009). Findings of Mohammadi *et al*., 2011) showed that application of biofertilizers had a significant effects on nutrient uptake of chickpea

2.6.1 Dual inoculations

Several workers have attempted to check the effect of dual inoculations of ABMs on plant growth and have noticed a positive plant growth response. Different combinations of ABMs such as the dual inoculation of symbiotic nitrogen fixing bacteria with asymbiotic nitrogen fixers, nitrogen fixers with P solubilizers or with PGPRs as well as combinations of strains among asymbiotic N fixers or P solubilizers or VAM fungi have also resulted in positive plant response. Dual inoculation of VAM fungus with symbiotic or asymbiotic N fixer or with a P solubilizer have shown positive plant response. Trials in field conditions also have improved the plant growth (Galal, 1997: Rodelas *et al*., 1999: Ramazon *et al*., 2004: Yadegari *et al*., 2008 and Askary *et al*., 2009). Synergistic interaction of microorganisms in dual inoculation seems to be the basic requirement for success of dual inoculations.

El-Komy (2005) demonstrated the beneficial influence of co-inoculation of *Azospirillum lipoferum* and *Bacillus megaterium* for providing balanced nitrogen and phosphorus nutrition of wheat plants. The inoculation with bacterial mixtures provided a more balanced nutrition for the plants and the improvement in root uptake of nitrogen and phosphorus was the major mechanism of interaction between plants and bacteria.

2.6.2 Multiple or mixed inoculations

Veena (1999) reported the maximum plant growth, biomass yield and nutrient uptake in sorghum in treatment receiving consortium of eight organisms comprising bacteria, fungi and actinomycetes which was almost equivalent to the application of 75 to 100 per cent of recommended chemical fertilizers dose. The field experiment conducted by Devananda (2000) revealed that maximum plant growth, yield and nutrient uptake in pigeonpea was obtained in combined inoculation of *Rhizobium*, *Azospirillum* sp. and *Pseudomonas striata*.

Thus mixed cultures or consortial formulations have considerable potential for controlling the soil microbiological equilibrium and thus providing a more favorable environment for plant growth and protection. However, selection of combination of microorganisms having compatibility among them and synergistic effect on plant growth and on soil nutrient management has to be considered in preparation and formulation of consortium.

Mixed inoculation of nitrogen fixing bacteria and arbuscular-mycorrhizal fungi have resulted in synergistic interaction effect that results in significant increase in plant growth, enhanced Mycorrhizal fungal infection, and an enhancement in the uptake of mineral nutrients such as phophorus, nitrogen and others (Chanway and Holl, 1991 and Linderman, 1997). Symbiotic nitrogen fixer and phosphate solubilizing microorganisms play an important role in supplementing nitrogen and phosphorus to the plant, allowing a sustainable use of nitrogen and phosphate fertilizers (Tambekar *et al.,* 2009).

Research efforts in these fields have steadily increased, resulting, in recent years, in the selection of numerous strains showing several beneficial features (Podile and Kishore, 2006 and Vessey, 2006].

2.7 Limitations of formulations of ABMs

Formulations of ABMs are in general available either as powdered or granular or liquid formulations (Smith, 1992). Powdered formulations are usually prepared using peat as carrier material and are coated on seeds using sticker material, however they seem to be unsuccessful always. It is mainly due to low population and low survival of inoculants on the seed surface and in rhizosphere soil. Though liquid formulations have been improvised by cell protectants to ensure better survival of ABMs, their effect on plant growth and survivability is not effective under varied soil conditions (Deaker *et al*., 2004; Hynes *et al.,* 1995, Hynes *et al.,* 2001). Other limitations of liquid formulations noticed are requirement of cool temperatures for storage, limited shelf life, increase in handling and end user costs. It is often perceived to be more expensive than the chemical fertilizers due to lack of skills and technology to produce biofertilizer products from abundant wastes. Besides, the effect on crops is slow, compared to chemical fertilizers. Special care such as storage or mixing with powders is also needed to handle microbial inocula to make they remain effective for extended use. As biofertilizers contain living organisms, their performance therefore depends on environment surrounding. Hence, outcomes are bound to be inconsistent.

A wider use of microbial inoculants, especially those acting as phytostimulators and biofertilizers, has been frequently hampered due to the variability and inconsistency of results between laboratory, greenhouse, and field studies. The reason for these discrepancies lies in the incomplete understanding of the complex relationships established between the components of the system: the plant, the microorganisms, and the environmental conditions, particularly that of soil (Artrusson *et al.,* 2006). In addition, the lack of correct formulations and the expensive and time consuming procedures of registration are also among the factors holding back the use of PGPM on a wider scale (Guillon, 2006).

2.8 Alginate based formulations of ABMs

Alginate bead encapsulation is known to protect inoculants from stress factors and release them gradually thus serving as viable inoculum source for a long period to facilitate their establishment in rhizosphere (Bashan *et al.,* 2002 and Vassilev *et al*., 2001). Further alginate beads are capable of entrapping sufficient number of bacterial cells (Fenice *et al.,* 2000 and Zohar-Perez *et al.,* 2002).

Alginate beads are mechanically stable and biodegradable which is a preferable trait of the formulation material. Bashan (1986) succeeded in preparing formulation of alginate with skim milk being a large reservoir of *Azospirillum* or *Pseudomonas* cells. and the release of bacterial cells from beads was at a slow and steady rate.

Interestingly it was noted that entrapment of living bacterial cells is efficient and their survival in alginate beads is better with the moderation of alginate concentration and addition of adjuvant material. The highest survival of bacterial cells was obtained by addition of skim milk and controlled dehydration of alginate beads (Fages, 1990). Strullus and Plenchette (1991) have stated that the physiological properties of mycorrhizal roots and vesicles were stabilized due to their entrapment in alginate beads. Ivanova *et al.,* (2005) studied encapsulation of nitrogen fixing *Azospirillum* in alginate and found that their application resulted in better yield than application of liquid and powdered formulations in field conditions.

Polymer formulations offer a long shelf life even at ambient temperature since they provide protection against environmental stresses and a consistent batch quality due to standardized production. Nevertheless, storage at cool temperature (4◦C) allows to maintain a longer viability of encapsulated cells (Bashan, 1998).

Thus alginate bead formulations can be considered for further development of consortial formulations of ABMs.

2.9 Consortial formulations of ABMs

Plant requirement is not limited to a single nutrient or to a phyto-hormone and that inoculation with various groups of ABMs could benefit the plant most. ABMs such as nitrogen fixers, phosphate solubilizers and plant growth promoters could improve crop growth and fertility status of agricultural fields. Combinations of these beneficial microorganisms have to be functioning effectively in soil to enhance yields sustainably. Combination of different interactive microorganisms is known as microbial consortia.

Use of ABMs in consortia could act together as a community and interact synergistically resulting in more efficient formulations than their single strain formulations. Further it is considered important to identify the best strain of beneficial microbes, verify their compatibility and combined efficacy both *in vitro* and *in vivo* and formulate consortia (Higa and Wididana, 1991).

Recently, the use of biofilms has also been proposed as possible means to produce effective plant inocula (Seneviratne *et al.,* 2008). A biofilm consists of microbial cells embedded into a self produced polymeric matrix (known as an extracellular polymeric substance - EPS) and adherent to an inert or living surface, which provides structure and protect ion to the microbial community. Three major types of biofilms can occur in the soil: bacterial (including Actinomycetes), fungal, and fungal-bacterial biofilms. Both bacterial and fungal biofilms are formed on abiotic surfaces, while fungi act as the biotic surface in formation of fungal bacterial biofilms (Seneviratne *et al.,* 2008). Majority of plant associated bacteria found on roots and in soil are forming biofilms (Ude *et al.,* 2006).

Hence consortial formulations are hypothesized to reduce operational cost and increase chances of improving crop growth and yield. Present work aims at formulating consortia of ABMs in alginate beads and to determine their response on plant growth.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

Investigations were conducted to develop alginate based consortial formulations of agriculturally beneficial microorganisms (ABMs) and to determine their effect on plant growth responses. These experiments were conducted at the Department of Agricultural Microbiology, Gandhi Krishi Vignana Kendra (GKVK), University of Agricultural Sciences, Bangalore.

3.1 Preparation of alginate beads with different plant nutrient cations

Alginate bead preparation with plant nutrient cationic compounds such as $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $CaCl_2$ were attempted with 2 per cent sodium alginate. Sterilized solutions of these compounds prepared at specific concentrations (0.050 M, 0.075 M, 0.100 M, 0.150 M and 0.200 M) were used to prepare beads, as per the procedure of Hegde and Brahmaprakash (1992). Observations on the nature of beads formed were recorded.

3.2 Optimization of concentrations of sodium alginate and calcium chloride for bead formation

This experiment was conducted to determine the optimum concentrations of sodium alginate and calcium chloride for alginate bead formation. Four different concentrations of sodium alginate (0.05 M, 0.1 M, 0.2 M and 0.3 M) and three different concentrations of calcium chloride (0.05 M, 0.1 M and 0.2 M) were tried in all possible combinations and the observations on nature of beads formed were recorded.

3.3 Screening of bacterial isolates

Bacterial isolates for this study were obtained from the department of agricultural microbiology, UAS, GKVK. To determine the extent of nitrogen fixed by selected cultures *viz Azotobacter* sp. and *Azospirillum* sp.,were grown in Waksmann-77 medium and sodium malate medium respectively. To determine the extent of phosphorus solubilization by *Bacillus* sp. And *Acinetobacter* sp. they were grown in modified Sperber's medium separately with North Carolina Rock Phosphate (NCRP) as a source of unavailable form of phosphorus (1.52 g/100ml broth). Amount of nitrogen fixed and P solubilized were estimated after seven days of incubation by determining the protein content in nitrogen and amount of soluble phosphorus in the filtrate of their broth cultures (Unkovich *et. al.,* 2008).

3.4 Selection of ABMs for consortial formulations

Principally a nitrogen fixer (*Azotobacter* sp.), phosphate solubilizer (*Acinetobacter* sp.) and plant growth promoting bacteria (*Pseudomonas* sp.) were considered for consortial formulation.

3.5 Development of consortial formulations

Bacterial cells from selected broth cultures of *Azotobacter* sp*., Acinetobacter* sp. and *Pseudomonas* sp. were harvested by centrifugation at 7826(×g) for 10 min at 4^{o} C. Pellets of bacterial cells obtained from centrifugation were re-suspended in the lowest possible volume of Ringer's solution(Prepared by dissolving 2.15g of sodium chloride, 0.075g of potassium chloride, 0.12g of Calcium chloride and 0.5g of sodium thiosulfate.$5H_2O$ in 1000 ml distilled water and pH adjusted to 6.6) and was mixed thoroughly with pre sterilized and cooled Na-alginate solution (2M). Resulting slurry was then dropped using pipette into pre-cooled and sterilized $CaCl_2$ solution (0.1 M). Formation of Ca-alginate beads were allowed to stabilize for 30 min in $CaCl_2$ solution itself. Beads were then washed 3 to 4 times with sterilized distilled water and were allowed to air dry in a laminar air flow.

3.6 Evaluation of the effect of consortial formulation on corn plant under greenhouse studies

3.6.1 Soil collection and processing

Soil used for this study was collected from an uncultivated field at GKVK campus, UAS, Bangalore. This soil is classified as kaolinitic, isohypothermic, kanhaplustalfs (SMSS technical monograph no. 19 USDA, 1990). Soil was sieved through a 4 mm sieve and was filled to plastic pots at the rate of 4 Kg per pot. Soil in the pots was mixed thoroughly to obtain a homogenous mixture. For the treatments that need autoclaved soil, soil was watered to 30 per cent of the moisture holding capacity and then subjected to autoclaving at a pressure of 1.06 kg/cm^2 and at 121^0C for one hour. These soil samples were autoclaved for the second time after eight days. Soil samples were subjected to three cycles of wetting and drying to attain equilibrium. At the end of each drying cycle, moisture content was raised to field capacity. Experimental treatments imposed to soil after these cycles of wetting and drying are explained below.

3.6.2 Fertilizer treatments

Recommended fertilization for corn is 150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of farm yard manure per hectare. Among these, inorganic nutrients were applied in the forms of NH_4NO_3, KH_2PO_4, K_2SO_4 and $ZnSO_4.7H_2O$, respectively at the rate of 0, 50 and 100 per cent of the recommended level. To ensure uniform distribution of nutrients in soil, nutrient solution with desired level of nutrients was prepared and applied. Treatments for which soil has to be amended with organic matter, well decomposed farm yard manure sieved through 2 mm sieve and sterilized in an autoclave was mixed with soil at 100 per cent recommended level. Soil samples were further subjected to three cycles of wetting and drying after application of nutrients.

3.6.3 Experimental layout

Green house experiment with corn consisted of 24 treatment combinations resulting from 2 soil conditions (Autoclaved and un-autoclaved), 2 organic matter amendments (-OM and +OM), 3 levels of chemical fertilization (0%, 50% and 100%) and 3 consortial formulations (C_0 and C_1). There were three replications for each treatment combination, constituting 62 experimental units. They were arranged in randomized complete block design on green house benches.

Consortial formulations used in this experiment were

C_0 – Uninoculated

C_1 –Consortia of *Azotobacter* sp., *Acinetobacter* sp. and *Pseudomonas fluorescens* in Ca-alginate beads

3.6.4 Seed treatment and planting of corn

Corn seeds of a composite cultivar (NAC-6002) were used in this study. Selected seeds were surface sterilized by treating with 70% alcohol for a minute and washed free of alcohol by rinsing them six times with sterilized distilled water. Seeds were soaked in water for an overnight period before planting. Five seeds were planted per pot and after emergence the number of seedlings was thinned to two per pot. Pots were maintained in greenhouse conditions for 50 days. The moisture content of soil in pots was maintained near field capacity by watering them with water passed through 45µm sieve.

3.7 Observations

3.7.1 Harvesting and determination of shoot and root dry weight

Plants were harvested at 50 days of sowing in case of corn considering that further growth of plants may not lead to growth differences due to treatments. Shoot portions were separated by cutting stems at the collar region. Roots were collected after washing them with a gentle stream of water. Shoot and root portions were dried separately in a hot air oven maintained at 60^0C. Dry weights of shoot and root were recorded after drying them to a constant weight. Total plant biomass was computed by summation of corresponding shoot and root dry weights of plants.

3.7.2 Nitrogen estimation in plant samples

Nitrogen concentration in selected shoot and root samples of plants was determined by Micro- Kjeldhal method as outlined by Jackson (1973). Plant samples (500 mg) were digested with digestion mixture (100:20:1 parts of K_2SO_4:$CuSO_4$:Se) and 20 ml of concentrated sulphuric acid at 400^0C till the solution becomes clear. The digest was cooled and diluted with distilled water. Digested samples were then distilled with 40 per cent sodium hydroxide and ammonia evolved was trapped in 4 per cent boric acid. Alkaline boric acid with mixed indicator (bromocresol green + methyl red) was titrated against 0.1 N sulphuric acid and volume of acid required for neutralization was recorded. A standard was run by distilling ammonium sulphate solution containing 5 mg of nitrogen and by back titrating it with 0.1N H_2SO_4. Nitrogen concentration in plant samples was calculated using the standard formula (Unkovich *et. al.,* 2008).

3.7.3 Determination of phosphorus in plant

Phosphorus concentration in selected shoot and root samples was determined by following the phosphomolybdate blue colour method (Murphy and Riley, 1962).

Known quantity of powdered plant samples were placed in test tubes and were subjected to dry ashing in a muffle furnace at 580^{o}C for 6hrs. ash was dissolved in 10ml of distilled water and colour was developed by adding 2.5 ml of reagent B(Reagent-B was prepared by dissolving 0.528 g of Ascorbic acid in 100 ml of Reagent-A. Reagent-A was prepared by dissolving 12 g of ammonium para molybdate and 0.2908 g of potassium antimony tartrate into 250 and 100 ml of distilled water, respectively to which 1000ml of 5N sulphuric acid was added and finally volume was made upto 2000ml with distilled water. This solution was stored in a dark coloured bottle and used when needed. The solution in test tube was mixed thoroughly. After 10 min the intensity of blue colour was measured by recording transmittance using UV-visible 1240 Spectrophotometer at 880nm. The phosphorus concentration was determined by comparing with a standard curve developed by using KH_2PO_4 as a source of P.

3.7.4 Potassium estimation in plants

Potassium in selected shoot and root samples was estimated by feeding the dry ashed plant sample in solution after suitable dilution to flame photometer (Jackson, 1973).

3.7.5 Nitrogen, phosphorus, potassium content in plant

Nitrogen content in plant samples was calculated using the nitrogen concentration and dry weight of shoot and root portions of plants. The total nitrogen content was obtained by summation of shoot and root nitrogen content. Similarly phosphorus and potassium contents of plant samples were calculated. The plant dry weight was the summation of shoot and root dry weight.

3.8 Statistical analysis

Data obtained from this experiment were subjected to analysis of variance by randomized complete block design using SAS programme version 9.2 and means were separated by the Duncan’s Multiple Range Test (DMRT) at 5 per cent level of significance (Little and Hills, 1978). The data on NPK concentration and their content were analyzed.

3.9 Effect of consortial formulation on *Leucaena* under greenhouse studies

3.9.1 Soil collection and processing

Soil collection and processing in this study was done as described earlier. Experimental treatments were imposed to soil after three cycles of wetting and drying.

3.9.2 Fertilizer treatments

Solution containing nutrients such as, K, Mg, Cu, B, Mo, Zn, Mn, and N in the form of KCl, $MgSO_4.7H_2O$, $CuSO_4.5H_2O$, H_3BO_3, $Na_2MoO_4.2H_2O$, $ZnSO_4$, $MnCl_2$, and Mg $(NO_3)_2.6H_2O$, respectively, which supply these nutrient elements at the rate of 100, 50, 5, 10, 0.5, 10, 5, and 34.6 mg per kg soil and P in the form of KH_2PO_4 at the rate of 100 mg per Kg soil which provides 0.12 mg P/l of soil solution was considered to be the 100 per cent recommended level of fertilization for *Leucaena leucocephala* plants along with farm yard manure at the rate of 10,000 kg per hectare. Accordingly different levels (0%, 50 %, 100% and 200%) of recommended fertilizers were imposed at the end of third cycle of wetting and drying. Soil was subjected to three more cycles of wetting and drying.

3.9.3 Experimental layout of the green house experiment with *Leucaena*

This experiment consisted of 24 treatments arising out of factorial combinations of 2 soil conditions (autoclaved and un-autoclaved), 2 levels of alginate (-Alginate and +Alginate), 3 levels of chemical fertilization (0%, 50% and 100%) and 2consortial formulations (C_0 and C_1). Three replicates were maintained for each treatment, thus constituting 62 experimental units in total. They were arranged in randomized complete block design on green house benches.

Consortial formulations used in this experiment were

C_0 – Uninoculated

C_1 –Consortia of *Azotobacter* sp. and *Acinetobacter* sp.

3.9.4 Seed treatment and planting of *Leucaena*

Seeds of *Leucaena* were scarified with concentrated H_2SO_4 for 15 minutes and washed free of acid by rinsing them six times with sterilized distilled water. The scarified seeds were germinated on water agar (0.9% agar) and three germinated seeds were planted per pot. After emergence seedlings were thinned to two per pot. Plants were grown for 65 days in greenhouse conditions and soil moisture was maintained at field capacity throughout the plant growth period.

3.9.5 Observations

Shoot dry weight, root dry weight and NPK content in plant tissues were recorded by following the procedures as described earlier. Plant biomass and NPK contents were calculated as explained in the previous experiment.

3.9.6 Statistical analysis

Data obtained from this experiment were subjected to analysis of variance by randomized complete block design using SAS programme version 9.2 and means were separated by the Duncan's Multiple Range Test (DMRT) at 5 per cent level of significance (Little and Hills, 1978).

3.10 Storage and determination of survivability of ABMs in consortial formulations

Consortial formulations were stored in refrigerated and under ambient conditions for 300 days. Population density in them was determined immediately after their preparation and after 300 days of their storage in refrigerated and in lab conditions. Bead formulations of consortium were dissolved in phosphate buffer of pH 6.8 by incubating overnight and by serial dilution plate count using respective culture media.

3.10.1 Preparation of phosphate buffer

Phosphate buffer of pH 6.8 was prepared by mixing 48 ml of 0.2 M potassium hydroxide (KOH) and 50 ml of 0.2 M potassium di-hydrogen orthophosphate (KH_2PO_4) solution and the volume was made up to 100 ml (Gomori, 1955).

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Experimental results of the research work taken up on consortial formulations of agriculturally beneficial microorganisms are presented in this chapter. Investigations were conducted to develop microbial consortial formulations of agriculturally beneficial microorganisms (ABMs) and to determine their effectiveness on plant growth in greenhouse conditions. Laboratory experiments were conducted to check alginate bead formation with different plant nutrient cations and to optimize the concentration of sodium alginate with calcium chloride for formulation. Screening of selected nitrogen fixing and phosphate solubilizing bacteria was taken up to determine their efficiency and for further selection. Alginate based inoculants formulation of consortia having selected agriculturally beneficial bacteria was prepared. Green house experiments were conducted to determine the effectiveness of these consortial formulations on plant growth in autoclaved and un-autoclaved soil conditions at different levels of recommended fertilization. The isolates of *Azotobacter chroococcum, Acinetobacter* sp. and *Pseudomonas fluorescens* used in this study were obtained from the culture collection of Department of Agricultural Microbiology, GKVK, UAS, Bangalore.

4.1 Development of Consortial formulations of ABMs

4.1.2 Alginate bead formation with different plant nutrient cations

In this experiment, bead formation with sodium alginate and different plant nutrient cation compounds such as $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $CaCl_2$ were studied. Varying concentrations of cationic compounds and sodium alginate were tried and the observations are presented in Table 1.

Table 1: Evaluation of alginate beads formation with different cations

Cationic compounds	Concentration of cationic compounds (M)					Sodium alginate (%)	Remarks on bead formation
	0.05	0.075	0.10	0.15	0.20		
$MgCl_2$	–	–	–	–	–	2	No bead formation
$MnCl_2$	–	–	+	+	+	2	Improper bead formation at concentrations > 0.1M
$ZnCl_2$	+	+	+	+	+	2	Soft bead formation at all the concentrations
$CaCl_2$	+	+	+	+	+	2	Soft beads at <0.1M and intact ones at >0.1M

Note: - No bead formation, + Bead formation.

Bead formation was good in combinations of sodium alginate with $CaCl_2$ as well as with $ZnCl_2$ at all the concentrations. However, with $MnCl_2$ beads were formed at concentrations >0.1M and were improper in shape. Bead formation was not noticed with $MgCl_2$ (Plate 1).

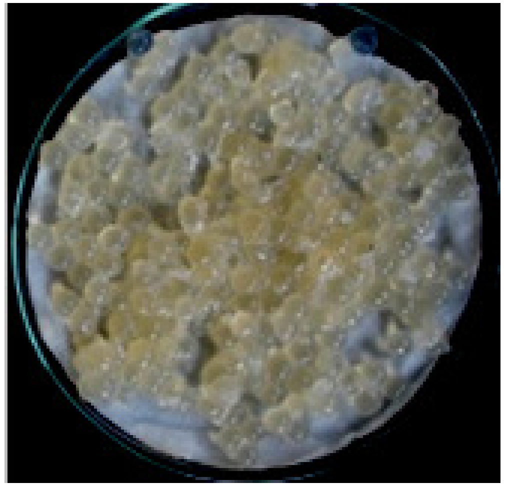
4.1.3 Optimizing the concentrations of sodium alginate and calcium chloride for bead formation

In this study four selected concentrations of sodium alginate (0.05 M, 0.1 M, 0.2 M and 0.3 M) and three concentrations of calcium chloride (0.05 M, 0.1 M and 0.2 M) were evaluated for their bead formation and stability. Selection of these concentrations was by based on the proportions of alginate and $CaCl_2$ used for alginate bead preparation in earlier works (Bashan, 1986 and Declerck *et al.,* 1996) and to further optimize their concentrations. Results are presented below (Table 2 and Plate 2).

Table 2: Alginate bead formation in different combinations of sodium alginate and calcium chloride concentrations

Concentrations of $CaCl_2$ (M)	Concentrations of Na-alginate (M)				Remarks)
	0.05	0.10	0.20	0.30	
0.05	–	–	–	–	No stable bead formation
0.10	–	+	+	+	Prominent & smooth beads at Na-alginate concentrations > 0.1M
0.20	–	+ +	+ +	+ +	Large & rigid beads at Na-alginate concentrations > 0.1M

Note: - No stable beads, + smooth and stable beads, + + Large and rigid beads



$MnCl_2$ (0.1 M)

$ZnCl_2$ (0.1 M)

$CaCl_2$ (0.1 M)

Plate 1. Alginate beads with different cationic compounds

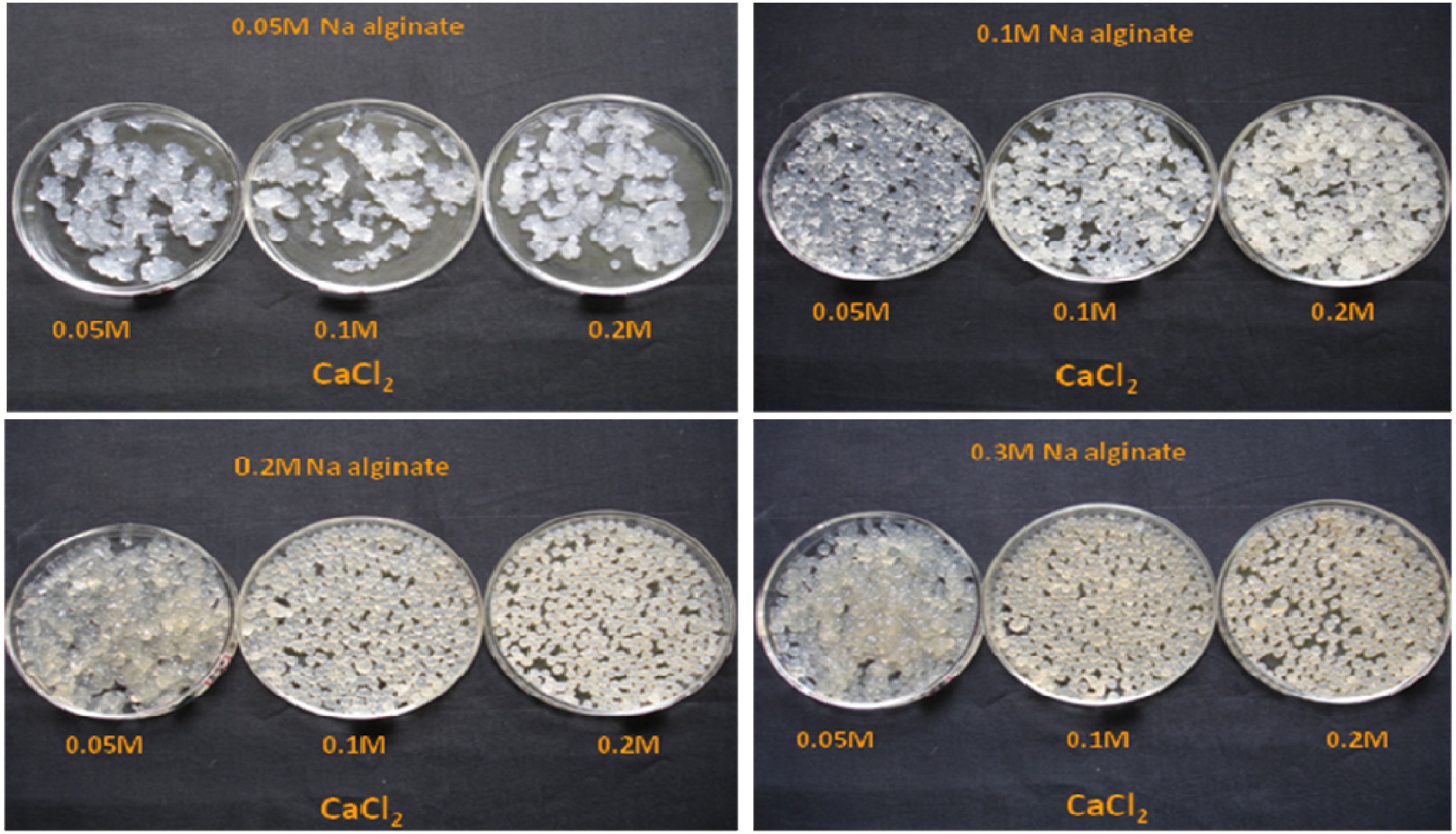


Plate 2. Optimization of alginate bead composition for consortial formulation of ABMs

4.2 Screening of ABM isolates

Bacterial isolates selected to check for their nitrogen fixing efficiency were *Azotobacter* sp. and *Azospirillum* sp. and that of the phosphate solubilization efficiency were *Acinetobacter* sp. and *Bacillus* sp. Amount of nitrogen fixed and phosphorus released by phosphate solubilization were determined after seven days of their growth in broth cultures. Results of this study are presented in Table 3.

Considering the observations, *Azotobacter* isolate was selected as an associative nitrogen fixer which fixed 8 mg of N per 100 ml of broth when compared to *Azospirillum* isolate which recorded 5.6 mg of N per 100 ml of culture broth. *Acinetobacter* isolate was selected as an efficient phosphate solubilizer which released 17.8 mg of P from a gram of NCRP compared to the *Bacillus* which released 17.1 mg of P. The selected isolates of nitrogen fixer and phosphate solubilizer along with *Pseudomonas fluorescense* as a plant growth promoting bacteria (PGPR) were used for further study. Compatibility of these isolates has been ensured by the cross streak method of Patel and Brown (1969).

4.3 Development of alginate bead consortial formulations of ABMs

Optimal concentrations of Na-alginate (2M) and 0.1 M solution of $CaCl_2$ were used to prepare alginate bead formulations. Bacterial cells collected by centrifugation from the broth cultures were dissolved at higher concentrations in Na-alginate solution to ensure sufficient population of them in resultant alginate beads (Plate 3).

Table 3. Mean values of nitrogen and phosphorus content in broth cultures

Bacteria	**N content (mg) /100ml broth**	**Bacteria**	**mg of P solubilized /g of NCRP***
Azotobacter sp.	8.0	*Bacillus* sp.	17.1
Azospirillum sp.	5.6	*Acinetobacter* sp.	17.8

*NCRP – North Carolina Rock Phosphate

Note: Mean values presented in the table are computed by three replicate values

Plate 3. Alginate bead formulations of ABMs

A. Alginate beads with *Azotobacter* sp. + *Acinetobacter* sp.

B. Alginate beads with *Azotobacter sp.* + *Acinetobacter sp.* + *Pseudomonas fluorescence*

4.4 Population density of microorganisms in alginate bead consortial formulations

Population density of bacteria in alginate beads were determined by serial dilution plate count method. The ABMs such as, *Azotobacter* sp.($37x10^9$), *Acinetobacter* sp.($54x10^9$) and *Pseudomonas* sp.($41x10^9$) were in the range of 10^9. The Bureau of Indian standards (BIS) has specified some standards with respect to the bioinoculant formulations according to which the minimum population density of an organism in the single strain bioinoculant formulation should be 10^7 per gram of the formulation. The standards for bioinoculant formulations with more than a strain has not been established so far and hence the standard of 10^7 population density specified for single strain bioinoculant formulations has been considered for reference and the population density in the consortial formulations prepared in this study are above this reference value (Table 4).

Table 4. Mean values of microbial population in alginate bead formulation

Bacteria	Population (CFUs/g of beads)
Azotobacter sp.	37×10^9
Acinetobacter sp.	54×10^9
Pseudomonas sp.	41×10^9

Note: Mean values presented in the table are computed by three replicate values

4.5 Green house study to evaluate the effect of consortial formulation on corn plant

Alginate bead consortial formulations of ABMs were evaluated for their plant growth response in green house conditions, using corn as the test plant. Green house experiment included the factorial combination of consortial formulations along with soil conditions and organic matter as well as inorganic nutrient amendments. The plants were grown for a period of 55 days in greenhouse conditions, maintaining the soil moisture at field capacity.

4.5.1 Nitrogen concentrations in shoot and root of corn

Main effects of treatments revealed that inoculation of soil with consortial formulation of ABMs significantly increased the nitrogen concentration in shoots (1.49%) as well as in roots (1.76%) of corn, compared to their uninoculated controls (1.15% and 1.39% respectively). Increase in nitrogen concentration of shoots due to other treatment factors such as soil sterilization and organic matter amendment had not significantly influenced the nitrogen concentration. Similar trend was noticed with respect to the nitrogen concentration of corn roots. Chemical fertilization at 0, 50 and 100 per cent of recommended fertilization had resulted in 0.76, 1.36 and 1.85 per cent of nitrogen concentration in shoots and that of 0.92, 1.49 and 2.32 per cent concentration of nitrogen in corn roots. The increase in nitrogen concentration due to different levels of chemical fertilization was significantly higher over their preceding concentrations of fertilization (Tables 5 and 6).

Interaction effect indicated that inoculation of soil with consortial formulation increased nitrogen concentration in shoots and roots compared to their uninoculated treatments, in conditions of soil either autoclaved or unautoclaved, either added with organic matter or not or amended with different levels of chemical fertilization.

4.5.2 Nitrogen content in corn plants

Estimation and analysis of nitrogen content in corn plants revealed that the main effect of inoculation treatment increased nitrogen content (70.8 mg/plant) significantly compared to their uninoculated counter parts. Soil sterilization didn’t influence the nitrogen content of corn plants significantly. Amendment with either organic matter or with chemical fertilization at different levels (50% and 100% of recommended fertilization) had significantly increased the nitrogen content of corn plants to the levels of 65.3 mg per plant, 56.5 mg per plant and 124.4 mg per plant respectively (Table7). Among the interaction effects, chemical fertilization showed differences in sterilized and in the absence of organic matter than in unfertilized and organic matter added conditions

Table 5. Nitrogen concentration in shoot of corn as influenced by microbial consortia with other treatment factors

Consortia	Shoot nitrogen concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.62 d	1.14 bc	1.57 a	0.70 d	1.22 b	1.60 d	0.68 d	1.06 c	1.63 a	0.71 d	1.20 bc	1.66 a
C_1	0.79 d	1.37 c	2.06 a	0.82 d	1.62 b	2.10 a	0.82 d	1.58 b	2.00 a	0.88 d	1.61 b	2.14 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Shoot nitrogen (%)
Consortia	C_0	1.15 b
	C_1	1.49 a
Soil sterilization	+	1.30 a
	-	1.33 a
Organic matter	-	1.28 a
	+	1.36 a
Fertilization	F1	0.76 c
	F2	1.36 b
	F3	1.85 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* *Acinetobacter* sp. and *Pseudomonas fluorescens*

Table 6. Nitrogen concentration in root of corn as influenced by microbial consortia with other treatment factors

Consortia	Root nitrogen concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.77 d	1.16 c	2.11 a	0.78 d	1.33 b	2.00 a	0.79 d	1.39 b	1.97 a	0.88 d	1.42 b	2.08 a
C_1	0.97 d	1.56 c	2.57 b	1.00 d	1.70 c	2.83 a	1.03 d	1.66 c	2.50 b	1.15 d	1.70 c	2.50 b

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Root nitrogen (%)
Consortia	C_0	1.39 b
	C_1	1.76 a
Soil sterilization	+	1.58 a
	-	1.57 a
Organic matter	-	1.54 a
	+	1.62 a
Fertilization	F1	0.92 c
	F2	1.49 b
	F3	2.32 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum, Acinetobacter* sp. and *Pseudomonas fluorescens*

Table 7. Nitrogen content in corn plant as influenced by consortial formulation of ABMs with other treatment factors

Consortia	Plant nitrogen content (mg/plant)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	9.2 f	47.9 de	105.1 b	12.0 f	93.3 c	157.4 a	12.1 f	37.1 def	71.1 c	21.4 ef	62.2 dc	122.6 b
C_1	13.6 f	57.3 de	142.6 bc	12.5 f	101.3 c	203.7 a	9.7 f	44.8 def	141.0 bc	22.9 ef	68.4 d	144.6 bc

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Plant nitrogen (mg/plant)
Consortia	C_0	54.8 b
	C_1	70.8 a
Soil sterilization	+	58.1 a
	-	52.7 a
Organic matter	-	46.3 b
	+	65.3 a
Fertilization	F1	13.1 c
	F2	56.5 b
	F3	124.4 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum, Acinetobacter* sp. and *Pseudomonas fluorescens*

4.5.3 Phosphorus concentration in shoot and root of corn

Corn plants grown in sterilized soil did not differ in their shoot and root phosphorus concentrations from those grown in unsterile soil. The organic matter amendment showed marginal increase in phosphorus concentration of shoot and roots. However, chemical fertilizer application at 50 and 100 per cent of recommended level and more importantly treatments of ABMs consortial formulation had significantly increased the phosphorus concentration in shoot and root portions of corn plants (Table 8 and 9).

Inoculation of soil with consortial formulation of *Azotobacter, Acinetobacter* and *Pseudomonas* had significantly increased phosphorus concentration in shoots and roots of corn plants grown in both sterilized and unsterile soils in general. These differences were noticed in plants grown in soils either with or without organic matter amendment and also in soils unfertilized or fertilized with inorganic nutrients (Table 8 and 9).

4.5.4 Phosphorus content in corn plants

Main effects of treatments indicated that the phosphorus content of corn plants grown in sterilized soil were higher than those grown in unsterile soil but the differences were not statistically significant. Such an increase was also noticed for addition of organic matter to the soil. Chemical fertilization showed significant increase in phosphorus content.

In general, the inoculation of soil with consortial formulation of ABMs showed increase in phosphorus content irrespective of soil condition and nutrient amendments. However, the differences were not statistically significant (Table 10).

Table 8. Phosphorus concentration in shoot of corn as influenced by consortial formulation of ABMs with other treatment factors

Consortia	Shoot phosphorus concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.20f	0.30cd	0.35b	0.23ef	0.30cd	0.37ab	0.21ef	0.28d	0.38ab	0.23e	0.32c	0.39a
C_1	0.25d	0.38c	0.48a	0.26d	0.41bc	0.49a	0.27d	0.39bc	0.48a	0.29d	0.42b	0.50a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Shoot phosphorus (%)
Consortia	C_0	0.30b
	C_1	0.39a
Soil sterilization	+	0.34 a
	-	0.35 a
Organic matter	-	0.33 a
	+	0.35 a
Fertilization	F1	0.24 c
	F2	0.35 b
	F3	0.43 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum, Acinetobacter* sp. and *Pseudomonas fluorescens*

Table 9. Phosphorus concentration in root of corn as influenced by consortial formulation of ABMs with other treatment factors

Consortia	Root phosphorus concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.23 d	0.33 c	0.45 ab	0.23 d	0.34 c	0.47 a	0.23 d	0.35 c	0.43 b	0.24 d	0.34 c	0.47 a
C_1	0.28 e	0.40 c	0.57 b	0.31 de	0.43 c	0.63 a	0.30 de	0.40 c	0.56 b	0.33 d	0.44 c	0.58 ab

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Root phosphorus (%)
Consortia	C_0	0.34 b
	C_1	0.43 a
Soil sterilization	+	0.39 a
	-	0.38 a
Organic matter	-	0.37 a
	+	0.40 a
Fertilization	F1	0.27 c
		0.038 b
	F2	0.51 b
	F3	0.69 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum, Acinetobacter* sp. and *Pseudomonas fluorescens*

Table 10. Phosphorus content in corn plant as influenced by consortial formulation of ABMs with other treatment factors

Consortia	Plant phosphorus content (mg/plant)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	2.7 g	13.2 def	18.9 d	3.5 g	20.3 dc	36.6 a	3.7 g	9.5 gef	26.1 bc	6.7 gf	16.1 de	27.6 b
C_1	4.4 d	15.5 c	37.0 b	4.4 d	29.7 b	46.8 a	3.0 d	10.6 cd	33.4 b	7.2 d	17.4 c	34.8 b

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Plant phosphorus (mg/plant)
Consortia	C_0	15.4 a
	C_1	20.3 a
Soil sterilization	+	19.4 a
	-	16.3 a
Organic matter	-	14.8 a
	+	20.9 a
Fertilization	F1	4.4 c
	F2	16.5 b
	F3	32.6 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum, Acinetobacter* sp. and *Pseudomonas fluorescens*

4.5.5 Potassium concentration in shoot and root of corn

Main treatment effects showed a significant increase in potassium concentration of shoot (1.38%) and roots (1.64%) of corn plants due to inoculation with consortial formulation of ABMs as compared to their uninoculated treatments (1.06% and 1.30% respectively). However, increase in treatments of soil sterilization and organic matter amendment was not significantly higher compared to their controls. Chemical fertilization at different levels (50% and 100%) has showed a distinct increase in potassium concentration of corn shoots (1.25% and 1.65%) and roots (1.40% and 2.06%) compared to their control treatments (0.95% in shoots and 0.76% in shoots) (Tables 11 and 12).

Interaction effects revealed that potassium concentration improved significantly in corn plant shoot and root portions for inoculation with consortia of ABMs in general irrespective of the soil autoclaved conditions as well for amendments with organic matter or with chemical fertilization.

4.5.6 Potassium content in corn plants

Main treatment effects revealed that inoculation with consortial formulation of ABMs had significantly increased the potassium content of corn plants (65.7 mg/plant) over their uninoculated counter parts (50.7 mg/plant). The increase in potassium content of corn plant was also noticed for treatment of organic matter amendment (60.5 mg/plant) compared to its control treatment (42.8 mg/plant) but the differences for soil sterilization treatment was not statistically different. Chemical fertilization at 50 and 100 per cent of recommended fertilizers had significantly increased the potassium content of corn plants to 52.4 mg per plant and 110.8 mg per plant compared to the unfertilized treatment with only 13.4 mg per plant. The interaction effects of consortial treatment with soil autoclaved conditions and that with organic and chemical nutrient amendments reveal that in general inoculation of consortia improves the potassium content of corn plants (Table13).

4.5.7 Plant biomass of corn plants

Interaction effects and main effects of soil sterilization, organic matter addition, chemical fertilization and inoculation of consortia of agriculturally beneficial microorganisms on biomass accumulation of corn plants are presented in Table 14a. Chemical fertilization at both 50 and 100 per cent levels and even addition of organic matter (20g/ pot /4kg soil) have significantly increased the corn plant dry matter accumulation. Soil inoculation with agriculturally beneficial bacterial consortium did not influence the biomass of corn.

Table 11. Potassium concentration in shoot of corn as influenced by microbial consortia with other treatment factors

Consortia	Shoot potassium concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.62 e	1.09 cd	1.37 b	0.70 e	1.12 c	1.40 ab	0.66 e	1.00 d	1.44 ab	0.73 e	1.13 c	1.48 a
C_1	0.78 d	1.30 c	1.84 a	0.87 d	1.43 bc	1.89 a	0.85 d	1.40 bc	1.85 a	0.91 d	1.51 b	1.93 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Shoot potassium (%)
Consortia	C_0	1.06 b
	C_1	1.38 a
Soil sterilization	+	1.20 a
	-	1.24 a
Organic matter	-	1.18 a
	+	1.26 a
Fertilization	F1	0.76 c
	F2	1.25 b
	F3	1.65 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter, Acinetobacter* and *Pseudomonas*

Table 12. Potassium concentration in root of corn as influenced by microbial consortia with other treatment factors

Consortia	Root potassium concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.82 d	1.22 c	1.77 ab	0.81 d	1.28 c	1.85 a	0.83 d	1.31 c	1.69 b	0.85 d	1.28 c	1.88 a
C_1	0.99 d	1.51 c	2.26 b	1.09 d	1.61 c	2.48 a	1.08 d	1.49 c	2.23 b	1.16 d	1.52 c	2.31 b

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Root potassium (%)
Consortia	C_0	1.30 b
	C_1	1.64 a
Soil sterilization	+	1.47 a
	-	1.46 a
Organic matter	-	1.43 a
	+	1.50 a
Fertilization	F1	0.95 c
	F2	1.40 b
	F3	2.06 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter, Acinetobacter* and *Pseudomonas*

Table 13. Potassium content in corn plant as influenced by consortial formulation of ABMs with other treatment factors

Consortia	Plant potassium content (mg/plant)											
	Sterilization (+)						Sterilization (-)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	9.4 f	47.5 cde	90.3c	12.2 f	87.4 b	140.1 a	12.4 f	35.0 def	61.8 c	21.5 ef	57.7 cd	109.8 b
C_1	13.6 e	54.7 d	126.9 bc	13.3 e	91.8 c	182.0 a	10.1 e	40.0 de	128.9 bc	23.4 e	63.1 d	131.3 bc

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Plant potassium (mg/plant)
Consortia	C_0	50.7 b
	C_1	65.7 a
Soil sterilization	+	53.8 a
	-	49.2 a
Organic matter	-	42.8 b
	+	60.5 a
Fertilization	F1	13.2 c
	F2	52.4 b
	F3	110.8 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter, Acinetobacter* and *Pseudomonas*

Table 14a. Plant biomass of corn as influenced by microbial consortia and other treatments

	Plant biomass (g/pot)											
	Sterilization (-)						Sterilization (+)					
	Organic matter (-)			Organic matter (+)			Organic matter (-)			Organic matter (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C0	1.29 f	4.18cde	5.13 bcd	1.51 f	6.41 b	9.24 a	1.65 f	3.10 def	6.72 b	2.77 ef	4.91 bcd	6.06 bc
C1	1.65 d	4.02 c	7.40 ab	1.53 d	7.11 ab	8.99 a	1.04 d	2.69 cd	6.61 b	2.32 cd	4.17 c	6.67 b

Means followed by the same letter within a row do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Root biomass (g/plant)
Consortia	C_0	8.82 a
	C_1	9.03 a
Soil sterilization	-	6.87 a
		6.09 a
	+	8.38 a
Organic matter		6.82 b
	-	
	+	8.99 a
Fertilization		3.17 c
		8.05 b
	F1	
	F2	
	F3	

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization
F2 – 50% of recommended chemical fertilization
F3 – 100% of recommended chemical fertilization (150 Kg of N, 75 Kg of P_2O_5, 40 Kg of K_2O, 10 Kg of Zn and 10,000 Kg of FYM per hectare)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter, Acinetobacter* and *Pseudomonas*

4.6 Green house experiment to study the effect of consortial formulation on *Leucaena*

Alginate bead consortial formulations of ABMs evaluated with corn as the test plant, led to the conclusion that the effect of consortial formulations on plant growth needs further clarification. So a second green house experiment was taken up using a dicot plant, *Leucaena,* to understand the effect of consortial formulation on plant growth and nutrient content. *Luecaena* is considered for this study as the dicot plant In this experiment, along with alginate based consortial formulations, alginate less consortial treatments were imposed. Pure broth cultures of organisms were used as alginate less treatments. Plants were grown in green house conditions by arranging the pots in completely randomized design (CRD). The plants were grown for a period of 65 days maintaining the soil moisture at field capacity.

4.7 Bacterial population density in alginate bead formulations

Population density of bacteria in Ca-alginate beads used for the green house experiment with *Leucaena* plants was determined by serial dilution plate count method. The population of ABMs, *Azotobacter chroococcum* (53 x 10^9) and *Acinetobacter* sp. (44 x 10^9) entrapped in Ca-alginate beads were in the range of 10^9. This level of population is higher than the population density recommended by BIS for bio inoculant formulations (10^7 per gram of the formulation) (Table 14b). Hence the alginate bead consortial formulations were taken for further green house study.

Table 14b. Bacterial population in alginate Beads

Bacteria	Population (CFUs/g of beads)
Azotobacter chroococcum	53 x 10^9
Acinetobacter sp.	44 x 10^9

Note: Mean values presented in the table are computed by three replicate values

4.7.1 Nitrogen concentration in shoot and root of *Leucaena*

The analysis of nitrogen concentration in shoots and roots of *leucaena* revealed that revealed that the treatments of consortial formulation inoculation, soil autoclaving and use of formulations without alginate have all not influenced the nitrogen concentration in both shoot and roots in comparison to their control treatments (Table 15 and 16). Treatments of 50 and 100 per cent chemical fertilization have significantly increased the nitrogen concentration in both shoots (1.51% and 1.91%) and roots (1.30% and 1.68%) respectively than the unfertilized control treatments. Increase in nutrient concentrations of plant tissues added with their chemical fertilizers is in known in general and the results of present experiment showed a similar trend. These trends were noticed in interaction effects as well as in main treatment effects.

4.7.2 Nitrogen content in *Leucaena* plants

Treatment main effects from analysis of total-N content in *Leucaena* plants revealed that inoculation with the consortial formulation of ABMs increased nitrogen content (52.0 mg/plant) compared to the uninoculated control (44.4 mg/plant). A similar higher N content in sterilized treatment (51.1 mg/plant) was recorded than the unsterilized control treatment (43.4 mg/plant) as well as chemical fertilization at 50 and 100 per cent of recommended quantity increased nitrogen content to 53.8 and 87.2 mg per plant compared to the unfertilized control treatment (20.7 mg/plant). The alginate factor didn’t affect the N content of *Leucaena* plants (Table-17).

4.7.3 Phosphorus concentration in shoot and root samples of *Leucaena*

Treatment main effects revealed that shoot and root P concentrations of *Leucaena* plants did not influence by soil sterilization and for the presence of alginate in formulations. The consortial formulation of ABMs increased the phosphorus concentration in shoots (0.40%) of *leucaena* marginally and that of roots (0.36%) significantly. In accordance with the general trend, chemical fertilization at 50 and 100 per cent of recommended quantity, significant increase in P concentration of shoot (0.39% and 0.43%) and root (0.36% and 0.39%) respectively were noticed (Table 18 and 19). Interaction effects revealed that the effect of inoculation of consortial formulation of *Azotobacter* and *Acinetobacter* on *Leucaena* was marginal irrespective of soil sterilization, presence of alginate in formulations and chemical fertilization of soil. Further optimization of factors to pronounce the effect of consortial formulations to significant levels requires specificity with respect to the plant and microbes involved

Table 15. Nitrogen concentration in shoot of *Leucaena* as influenced by microbial consortia in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Shoot nitrogen concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	1.14 c	1.46 b	1.82 a	1.12 c	1.57 b	2.00 a	1.09 c	1.43 b	1.85 a	1.08 c	1.44 b	1.84 a
C_1	1.22 c	1.54 b	1.97 a	1.19 c	1.61 b	2.00 a	1.03 c	1.57 b	1.91 a	1.12 c	1.55 b	1.87 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Shoot nitrogen (%)
Consortia	C_0	1.49 a
	C_1	1.55 a
Soil sterilization	+	1.55 a
	-	1.48 a
Alginate	-	1.50 a
	+	1.54 a
Fertilization	F1	1.12 c
	F2	1.51 b
	F3	1.91 a

Means followed by the same letter within a main effect do not significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization(K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

Table 16. Nitrogen concentration in root samples of *Leucaena* as influenced by microbial consortia in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Root nitrogen concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.77 e	1.08 cd	1.63 a	0.85 e	1.30 b	1.57 a	0.82 e	1.22 bc	1.74 a	0.90de	1.27 bc	1.52 a
C_1	0.91 c	1.30 b	1.76 a	0.88 c	1.33 b	1.70 a	0.84 c	1.40 b	1.76 a	0.91 c	1.44 b	1.73 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Root nitrogen (%)
Consortia	C_0	1.22 a
	C_1	1.33 a
Soil sterilization	+	1.26 a
	-	1.30 a
Alginate	-	1.27 a
	+	1.28 a
Fertilization	F1	0.85 c
	F2	1.30 b
	F3	1.68 a

Means followed by the same letter within a main effect do not significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

Table 17. Nitrogen content in plant of *Leucaena* as influenced by microbial consortia in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Plant nitrogen content (mg/plant)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	21.0 e	48.5 d	91.0 a	19.8 e	53.4 d	93.3 a	15.0e	47.4 d	78.7 b	18.1 e	49.4 d	64.3 c
C_1	21.2 e	56.8 d	110.1 a	27.4 e	64.2 cd	112.3 a	16.9 e	60.0 d	86.3 b	21.7 e	57.8 d	74.6 c

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Plant nitrogen (mg/plant)
Consortia	C_0	44.4 b
	C_1	52.0 a
Soil sterilization	+	51.1 a
	-	43.4 b
Alginate	-	47.1 a
	+	47.4 a
Fertilization	F1	20.7 c
	F2	53.8 b
	F3	87.2 a

Means followed by the same letter within a main effect do not differ differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

Table 18. Phosphorus concentration in shoot of *Leucaena* as influenced by consortial formulation of ABMs in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Shoot phosphorus concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.34 ef	0.40 bcd	0.40 bcd	0.35 e	0.40 bcd	0.44 a	0.31 f	0.38 d	0.41 bc	0.33 ef	0.38 cd	0.42 b
C_1	0.36 e	0.41 cd	0.44 abc	0.36 e	0.41 cd	0.46 a	0.32 f	0.39 d	0.45 ab	0.35 ef	0.41 cd	0.43 abcd

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Shoot phosphorus (%)
Consortia	C_0	0.38 a
	C_1	0.40 a
Soil sterilization	+	0.40 a
	-	0.38 a
Alginate	-	0.39 a
	+	0.40 a
Fertilization	F1	0.34 a
	F2	0.39 b
	F3	0.43 c

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

Table 19. Phosphorus concentration in root of *Leucaena* as influenced by consortial formulation of ABMs in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Root phosphorus concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.27 e	0.33 bcd	0.35 ab	0.29 cde	0.36 ab	0.37 ab	0.28 de	0.33 bcd	0.39 a	0.30 cde	0.34 abc	0.36 ab
C_1	0.31 c	0.36 b	0.40 ab	0.32 c	0.37 ab	0.39 ab	0.29 c	0.37 ab	0.41 a	0.31 c	0.37 ab	0.41 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Root phosphorus (%)
Consortia	C_0	0.33 b
	C_1	0.36 a
Soil sterilization	+	0.34 a
	-	0.35 a
Alginate	-	0.34 a
	+	0.35 a
Fertilization	F1	0.30 c
	F2	0.36 b
	F3	0.39 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

4.7.4 Phosphorus content in *Leucaena* plants

Main treatment effects indicated that the phosphorus content of L*eucaena* plants did not significantly influence by soil sterilization treatment. Presence of alginate in the consortial formulation also did not significantly influence the phosphorus content when compared to the phosphorus content of *Leucaena* plants grown without alginate in the formulation. Chemical fertilization to the extent of 50 and 100 of recommended quantity (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil) showed significant increase of 14.1 and19.8 mg per plant in phosphorus content respectively of *Leucaena* plants. Interaction effects reveal that inoculation of soil with consortial formulation of ABMs showed significant increase in phosphorus content in general (Table 20).

4.7.5 Potassium concentration in shoot and root samples of *Leucaena*

Main treatment effects showed a marginal increase in potassium concentration of shoot and roots of *Leucaena* plants for inoculation with consortial formulation of ABMs and similar trend was noticed in treatments with soil sterilization and alginated formulations. Chemical fertilization show a significant increase in potassium concentration of *Leucaena* shoots and roots (Tables 21 and Table 22).

The interaction effects revealed that potassium concentration was not influenced in *Leucaena* shoot and root tissues for the inoculation treatment with consortial formulation of ABMs in general.

4.7.6 Potassium content in *Leucaena* plants

Main treatment effects revealed that inoculation with consortial formulation of ABMs had significantly increased potassium content (48.3 mg/plant) of plants compared to the uninoculated treatments (41.2 mg/plant). Presence of alginate in consortial formulations have not influenced the potassium content of *Leucaena* plants. Chemical fertilization by 50% and 100% of recommended quantity has significantly increased the potassium content of *Leucaena* plants by 50.1 and 77.8 mg per plant respectively, compared to the unfertilized treatment (21.0 mg/plant). The interaction effects showed that inoculation with consortial formulation of ABMs has increased the potassium content of *Leucaena* plants in general irrespective of the soil sterilization and chemical fertilization (Table 23).

Table 20. Phosphorus content in Plant of *Leucaena* as influenced by consortial formulation of ABMs in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Plant phosphorus content (mg/plant)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	6.3 a	13.6 b	19.9 b	6.3 b	13.8 b	20.8 a	4.4 a	12.6 b	17.5 b	5.6 a	13.1 b	14.8 b
C_1	6.6 a	15.3 a	24.7 a	8.7 a	16.7 a	25.8 a	5.4 a	15.1 a	20.3 a	6.9 a	15.2 a	17.3 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Plant phosphorus (mg/plant)
Consortia	C_0	11.4 b
	C_1	13.6 a
Soil sterilization	+	13.3 a
	-	11.3 b
Alginate	-	12.1 a
	+	12.5 a
Fertilization	F1	6.5 c
	F2	14.1 b
	F3	19.8 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

Table 21. Potassium concentration in shoot of *Leucaena* as influenced by microbial consortia in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Shoot potassium concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	1.12 b	1.41 a	1.60 a	1.19 a	1.43 a	1.76 a	1.03 a	1.35 a	1.62 a	1.11 a	1.34 a	1.65 a
C_1	1.20 a	1.45 a	1.76 a	1.18 a	1.42 a	1.81 a	1.08 a	1.38 a	1.77 a	1.15 a	1.44 a	1.70 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Shoot potassium (%)
Consortia	C_0	1.38 a
	C_1	1.44 a
Soil sterilization	+	1.44 a
	-	1.39 a
Alginate	-	1.39 a
	+	1.43 a
Fertilization	F1	1.13 c
	F2	1.40 b
	F3	1.71 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

Table 22. Potassium concentration in root of *Leucaena* as influenced by microbial consortia in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Root Potassium concentration (%)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	0.81 a	1.13 a	1.36 a	0.87 a	1.25 a	1.44 a	0.84 a	1.14 a	1.49 a	0.88 a	1.15 a	1.38 a
C_1	0.91 a	1.25 a	1.54 a	0.95 a	1.26 a	1.49 a	0.87 a	1.27 a	1.57 a	0.91 a	1.28 a	1.59 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Root Potassium (%)
Consortia	C_0	1.15 a
	C_1	1.24 a
Soil sterilization	+	1.20 a
	-	1.19 a
Alginate	-	1.18 a
	+	1.21 a
Fertilization	F1	0.88 c
	F2	1.22 b
	F3	1.48 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

Table 23. Potassium content in plant samples of *Leucaena* as influenced by consortial formulation of ABMs in combination with treatments factors such as soil sterilization, soil fertilization and presence of alginate

Consortia	Plant Potassium content (mg/plant)											
	Sterilization (+)						Sterilization (-)					
	Alginate (-)			Alginate (+)			Alginate (-)			Alginate (+)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
C_0	20.5 a	47.7 a	79.2 b	20.9 b	49.1 b	82.7 b	14.4 a	44.7 b	68.5 b	18.4 a	45.8 a	57.9 a
C_1	20.9 a	53.7 a	98.0 a	27.7 a	57.5 a	101.1 a	17.7 a	53.1 a	79.2 a	22.2 a	53.2 a	68.0 a

Means followed by the same letter within a row, do not differ significantly at p=0.05 by LSD.

Mean values of Main effects

Main effects		Plant Potassium (mg/plant)
Consortia	C_0	41.2 b
	C_1	48.3 a
Soil sterilization	+	47.7 b
	-	40.5 a
Alginate	-	43.8 a
	+	44.1 a
Fertilization	F1	21.0 c
	F2	50.1 b
	F3	77.8 a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

C_0 – Uninoculated

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

4.7.7 Plant biomass of *Leucaena* plants

The Interaction effects of consortial formulation with soil sterilization, alginated formulation and with different levels of chemical fertilization on plant biomass of *Leucaena* plants are presented in Table 24. The inoculation of consortial formulation has significantly increased the plant biomass at all three levels of Chemical fertilization (0%, 50% and 100%) to the extent of 4.37, 7.93 and 9.92 grams per plant compared to their uninoculated controls 2.89, 6.45 and 8.63 grams per plant respectively. Thus inoculations with consortial formulations of ABMs are indicating a positive plant growth response in *Leucaena* plants as well.

Table 24. Biomass of *Leucaena* plants as influenced by alginate bead consortial formulations of ABMs

Treatments	Plant biomass (g/plant)						
	Sterilization		Alginate		Fertilization		
	-	+	-	+	F1	F2	F3
C_0	5.19^b	6.22^a	5.76^a	5.65^a	2.89^b	6.45^b	8.63^b
C_1	6.55^a	7.43^a	6.78^a	6.84^a	4.37^a	7.93^a	9.92^a

Means followed by the same letter within a main effect do not differ significantly at p=0.05 by DMRT.

C_0 - Un-inoculated control

C_1 – Inoculated with consortia of *Azotobacter chroococcum* and *Acinetobacter* sp.

F_1 – 0% of recommended chemical fertilization

F2 – 50% of recommended chemical fertilization

F3 – 100% of recommended chemical fertilization (K, Mg, Cu, B, Mo, Zn, Mn, N and P @ 100, 50, 5, 10, 0.5, 10, 5 34.6 and 0.12 mg per kg soil)

4.8 Storage and determination of survivability of ABMs in consortial formulations

Population density of bacteria in alginate bead consortial formulations was determined immediately after their formulation ($91x10^9$ *Azotobacter chroococcum*, $83x10^9$ *Acinetobacter* sp. and 88×10^9 *Pseudomonas fluorescens* CFUs per gram of bead). The beads were stored in refrigerated and in ambient temperature conditions for 300 days. Population density after 300 days of their storage was also determined. The results are presented in the table below (Table 25).

Results indicated that population density of bacteria in alginate beads were higher than 10^8 CFUs (Table 25). According to the BIS standards the single strain bioinoculant formulations should have a minimum population density of 10^7 per gram of the formulation. The standards for bioinoculant formulations with more than a strain has not been established so far and hence the standard of 10^7 population density specified for single strain bioinoculant formulations has been referred as the standard and thus the population density in the consortial formulations prepared in this study are considered to be above the standard recommended by BIS.

Table 25. Population of ABMs in consortial formulation

Population (CFUs)/g of beads			
Bacteria	In fresh beads	Stored in refrigerated conditions for 300 days	Stored in ambient conditions for 300 days
Azotobacter chroococcum	91×10^9	56×10^9	53×10^8
Acinetobacter sp.	83×10^9	39×10^9	44×10^9
Pseudomonas fluorescens	88×10^9	82×10^8	51×10^8

Note: Mean values presented in the table are computed by three replicate values

DISCUSSION

V. DISCUSSION

The direct involvement of soil microorganisms is influencing plant growth and development, through processes such as nutrient transformation, nutrient uptake, biological control and production of growth promoting substances are well known. However, it appears that their potential under field conditions is yet to be realized. Major limitation for this is ineffectiveness of microbial formulations in soil system where native micro flora act antagonistically with the introduced ones. Further, use of bio-fertilizers is limited due to limitations such as reduced shelf life in storage conditions, inconsistent growth responses caused by abiotic stress factors such as higher temperatures during storage, drought or water stagnation in field conditions. An alternative to this could be the development of consortial formulations with beneficial microorganisms having different physiological capabilities to sustain their activity in wide range of field conditions.

Entrapment into natural polymers such as alginate and their introduction to soil is noticed to provide the organisms protection from the native soil microflora. Further, enabling them to interact and finally develop a stable microbial community in the rhizosphere. This could enable them to have higher chances of establishing in soil and cause desirable effect on plant.

Present investigation was conducted to develop alginate based consortial formulations of agriculturally beneficial microorganisms and to evaluate their effect on plant growth in green house. Results of the investigation are discussed in this chapter, in the context of developing more effective alginate based consortial formulations of ABMs for field application.

5.1 Development of consortial formulations of ABMs

5.1.1 Alginate beads with plant nutrient cations

Alginate bead preparations attempted with different plant nutrient cations revealed that intact bead formation was suitable for consortial formulation of ABMs and soil application was possible with 0.1M $CaCl_2$ and 2M sodium alginate among the various concentrations of different cationic compounds evaluated. The other compounds such as $MnCl_2$ and $ZnCl_2$ formed beads but they were neither uniform nor stable. Differences with respect to beads formation of these cations was possibly due to differences in their ionic properties. The review of literature revealed that attempts to evaluate the bead preparation of these cations with alginate have not been carried out in earlier works. Hence, 0.1M $CaCl_2$ and 2M sodium alginate were considered for development of consortial formulations which are supposed to retain the entrapped inoculants.

5.1.2 Selection of ABMs to constitute consortia

Among the major plant growth promotional activities happening in natural ecosystems with the assistance of micro organisms, the prominent ones are nitrogen fixation by symbiotic and asymbiotic nitrogen fixing bacteria in addition to phosphate solubilizing and plant growth promoting rhizo bacteria which are known to enhance the inoculation effect on plant growth in combination with other organisms (Yadegari, *et al*. 2008: Askary, *et al*. 2009). Thus a combination of these organisms is considered to constitute the microbial consortial formulation. Isolates of *Azotobacter* and *Azospirillum* which are free living nitrogen and associative nitrogen fixers, isolates of *bacillus* and *Acinetobacter* which are known to solubilize rock phosphate and plant growth promoting *Pseudomonas* are considered.

5.1.3 Screening of isolates for their efficiency

Among the isolates selected and screened, *Azotobacter* showed a higher nitrogen fixing activity (8mg of N/100ml of broth culture) than *Azospirillum* and *Acinetobacter* showed a higher rock phosphate solubilizing activity (17.8mg of P solubilized /g of NCRP) than *Bacillus.* For preparation of consortial formulation of ABMs, combination of a nitrogen fixer, a phosphate solubilizer and a PGPR microorganism was considered to be more ideal and was considered to influence the plant growth more effectively by augmenting for the major nutrient and plant growth promoting activity. Hence, *Azotobacter* for nitrogen fixation and *Acinetobacter* for phosphate solubilization were considered to constitute consortial formulation along with *Pseudomonas fluorescens* as a plant growth promoting rhizobacteria (PGPR).

5.1.4 Development of protocols for alginate bead formulation of consortia

Protocols to prepare alginate bead formulations of microbial consortia were developed by optimizing the concentrations of Na-alginate (2M) and $CaCl_2$ (0.1M) for inoculum entrapment and bead formation. Work on alginate bead formation and standardization of protocols has been attempted by other workers also (Bashan, 1986.) Procedure to obtain concentrated inoculum suspension from independently grown broth cultures of different isolates was determined to be done by centrifugation and re-suspension of the pellets in saline solution at higher concentrations for entrapping them in Ca-alginate beads. The population density of microorganisms in consortial formulations was verified by serial dilution plating and the population density of each organism in consortia was ensured to be at a level higher than the recommended (10^7) before using the formulation for further studies.

5.2 Green house studies to evaluate the effect of consortial formulation on plant growth and nutrition

Digat (1993) with his new encapsulation technique proposed that mixed microbial cultures in the encapsulated capsule allow their components to interact with each other synergistically and indicated that consortial formulations could result in positive effect. Though such attempts to develop formulations containing more than one organism have been made, their effect on plant response has not been studied. Present investigation was taken up to demonstrate the effect of consortial formulations of ABMs on plant growth and nutrition.

5.2.1 Effect of alginate based consortial formulations on plant nutrition in different soil conditions and with different soil amendments

The effect of consortial formulation on growth of corn and *Leucaena* plants were studied in sterilized and unsterilized conditions of soil and with different levels of chemical fertilization (0%, 50% and 100%). Further, the effect of these formulations when soil is added with organic matter and when alginate is present with the formulation was studied. Inoculation of soil with consortial formulation of ABMs has significantly increased the nutrient concentrations (N, P and K) in shoot and root portions of both corn and *Leucaena* plants, in addition to the increase in plant biomass. This effect of consortial formulation on plant growth and nutrient concentrations in comparison to their uninoculated counter parts is mainly considered to be due to the microbial activity resulted from inoculation of consortial formulation. Sterilization of soil has marginally increased plant biomass and nutrient levels of plants, which is presumed to be due to dissolution of salts and organic matter at higher autoclaving temperatures and pressure. Chemical fertilization has significantly increased the nutrient concentrations in shoot and root portions of both the plants which is known to be due to supplying of plant nutrients mainly in readily usable form to soil and that way to the plant root system. .

Results of the present study revealed that alginate bead formulations of ABMs containing bacteria are effective in improving plant growth and nutrient uptake. Similar improvement in plant growth response was noticed in an earlier study taken up by Bashan (1986). Alginate bead formulations of *Azotobacter* and *Pseudomonas* inoculated to wheat plants under field conditions survived in the field long enough and their populations were comparable to the survival of bacteria originating from peat-based inoculants and were known to have more survival rate than the non encapsulated formulations (Bashan, 1986 and Trevors et al., 1992). Inoculum of *Pseudomonas* prepared by encapsulating with alginate showed decrease in cell numbers moderately in soil whereas the non encapsulated free inoculum showed large reduction in bacterial numbers (Bashan, 1998). These studies provide clear evidence that alginate beads are the carriers for plant inoculants which provide a protective environment in the soil. In this context, results of the present study suggest that nitrogen fixation by function of nitrogen fixing bacteria, P solubilization by phosphate solubilizing bacteria and K mobilization by plant growth promoting bacteria could be the major mechanisms causing increase in N, P and K uptake both in corn and *Leucaena* (Thomas, 1993; Fan *et al.* 2011 and Minorsky, 2008).

The effect of inoculation of microbial consortia of bacteria could be slow due to delayed release of entrapped inoculants from consortial formulations, as the dissolution of beads and release of entrapped bacteria takes some time depending on the soil conditions (Bashan, 1986). Microbial activity is known to be enhanced in rhizosphere due to root exudates (Lakshmi Kumari, 1961), delay in the release of entrapped inoculants from the alginate matrix may reduce the chances of alginate entrapped organisms releasing to the rhizosphere and establishing a niche there. Initial low populations of bacteria in the rhizosphere in case of alginate based formulations have been observed to be due to slow release of entrapped bacteria from the alginate beads (Bashan, 1986). Early degradation of beads is noted to be essential to release entrapped microbial cells to soil and colonize the plant rhizosphere for optimum nodulation and nitrogen fixation in case of *Bradyrhizobium japonicum* (Brockwell *et al*., 1980).

5.2.2 Plant growth response in greenhouse conditions for different soil treatments

Marked increase in shoot, root and total plant biomass accumulation was noticed in these studies for soil sterilization, organic matter addition, alginate less inoculum and chemical fertilization. Increase in plant biomass due to sterilization seems to be mainly due to increased concentration of soluble P in autoclaved soil as autoclaving the soil is known to significantly increase soluble P to the extent of 60 per cent (Anderson and Magdoff, 2005). Further, increase in biomass of corn and *leucaena* after sterilization was attributed to sterilization effect of soil, which killed native micro flora and assisted for plant growth and nutrient uptake (Wu *et al.,* 2005). Sterilization of soil by autoclaving is also known to significantly increase concentrations of water soluble organic carbon as well (shaw *et al.,* 1999).Increase in biomass of corn for organic matter addition observed in this investigation was similar to the trend noticed earlier by Wu *et al.,* (2005).

Increase in plant biomass for chemical fertilization is a well known trend which was observed in this experiment but the toxicity of chemical fertilization observed in this experiment shows that the optimum level of fertilization set for the *Leucaena* seems to be optimal and plant growth has severely affected by the toxicity of higher level of chemical fertilization.

Results of this study indicated that the alginate consortial formulations of ABMs containing bacteria are effective in promoting plant growth and nutrient uptake.

5.3 Storage and survivability of ABMs in alginate based consortial formulations

The protocol developed to prepare alginate bead consortial formulations of ABMs was successful in maintaining the population of constituent organisms above the level recommended in general (10^7). Storage of these formulations in refrigerated and in ambient temperature has shown negligible reduction in population density of constituent microorganisms up to 300 days. Considering these results it is concluded that the shelf life of consortial formulations developed in this study is satisfactory up to 300 days either at refrigerated or at ambient temperatures and thus constitute effective consortial formulation of ABMs that improve plant growth.

Thus it is concluded that formulation of microbial consortia of selected ABMs is possible and their effect on plant growth improvement is positive. Further, future studies have to focus on the specificity with respect to its constituent organisms and plant and soil conditions in which they benefit plants significantly.

SUMMARY

VI. SUMMARY

Consortia of agriculturally beneficial microorganisms were formulated in alginate beads and their effect on plant growth was determined. ABMs selected to constitute consortia were *Azotobacter chroococcum*, *Acinetobacter* sp. and *Pseudomonas fluorescence.* Population density of each constituent organism in the consortial formulation was ensured to be above the recommended level (10^7/g). Storage of alginate based consortia in refrigerated and in lab conditions retained population density if constituent organisms over a period of 300 days.

Effect of consortial formulation on growth response of corn (*Zea mays* L.) was studied in green house conditions. Selected seeds of the composite cultivar (NAC-2006) of corn were surface sterilized and soaked in water for an overnight period before sowing. There were 24 treatments resulting from factorial combination of 2 soil conditions, 2 organic matter amendments, 3 levels of chemical fertilization and 2 consortial formulations. With three replicates for each treatment, totally 62 experimental units were maintained.

Soil used was homogenized in general, but autoclaving, nutrient amendment and microbial inoculation was done according to the treatment requirements. Plants raised in green house conditions were maintained at soil moisture near field capacity. After 55 days of their growth, shoots were harvested. Total plant biomass was derived by the summation of corresponding shoot and root dry weights. Results were subjected to statistical analysis. Plant response was positive for consortial treatments of bacteria with respect to nutrient concentrations and content of experimental shoot and root tissues at main effect level.

The plant selected was *Leucaena.* In this experiment 24 treatments were designed from the factorial combination of 2 soil conditions, 2 alginate conditions, 3 levels of chemical fertilization and 2 consortial formulations. With three replicates for each treatment, totally 62 experimental units were maintained. Even these plants were raised in green house conditions maintaining moisture content of the soil near field capacity. After 65 days of their growth, shoots were harvested. Roots were separated from the pots by washing them free of soil. Root portions were placed in an oven for drying. The biomass of shoots and roots were noted after drying them to a constant

weight at 60^0C. Total plant biomass was computed as the sum total of corresponding shoot and root dry weights. Nutrient concentrations of nitrogen, phosphorus and potassium for selected treatments were determined in shoot and root samples of *Leucaena*. Their results were subjected to statistical analysis.

Results of the green house experiment with *Leucaena* revealed that consortial formulations increased nutrient content of N, P and K in shoot and root tissues of *Leucaena* as well as in their content and biomass.

All these observations give an impression that formulations of consortia of ABMs can be prepared in alginate beads. But there is need to screen for the consortial combination of organisms that result in significant improvement in plant growth. Soil conditions and plants with which a consortial formulation is effective need to be determined. As rhizobial inoculation is effective in legumes and as VAM fungal inoculation is effective in certain dependent plant species and soil P conditions, a consortium of ABMs could be more effective for a specific plant species and in certain set of soil conditions with respect to its nutrient status and physico-chemical properties.

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VII. REFERENCES

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