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Development of Hydrogel based Bio-Inoculant Formulations and their Impact on Plant Biometric Parameters of Wheat (*Triticum aestivum* L.)

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A B S T R A C T

The green revolution bought amazing consequences in food grain production but with insufficient concern for agriculture sustainability. Biofertilizers are gaining importance in sustaining agriculture. Various complementing combinations of microbial inoculants for management of major nutrients are necessary for agriculture sustainability. The present investigation was conducted to optimize the effect of hydrogel for their ability to support growth, shelf life stability and bio-efficacy of hydrogel based bioinoculants (*Azotobacter chroococcum, Pseudomonas fluorescence* and *Trichoderma viride*). Hydrogel products constitute a group of polymeric materials, the hydrophilic structure of which renders them capable of holding large amounts of water in their three-dimensional networks. Extensive employment of these products in a number of industrial and environmental areas of application is considered to be of prime importance. Shelf life studies of bio-inoculants formulations with three different carriers *viz*: lignite, liquid and hydrogel shelf after 90 days incubation at room temperature, exhibited population of nitrogen fixer *Azotobacter chroococcum* 1.2×10^7, 1.4×10^8 and 3.5×10^9 CFU mL^{-1}, phosphate solubilizer *Pseudomonas fluorescence* 2.2×10^7, 2.4×10^8 and 4.5×10^9 CFU mL^{-1} and phyto-stimulator *Trichoderma viride* 1.4×10^6, 2.8×10^7 and 2.5×10^8 CFU mL^{-1} respectively. Wheat seeds treated with hydrogel based bioinoculants and the consortium enhanced plant growth positively by a multiplicity of synergistic mechanism compared to liquid and lignite based microbial consortia.

Introduction

Biofertilizers are living microorganisms; they themselves are not source of nutrients but can help the plant in accessing the nutrient available in its surrounding environments (Suman *et al.*, 2015).

In general, commonly microbes used as biofertilizers may be *Azotobacter*, *Azospirillum*, *Rhizobium* as nitrogen fixing soil bacteria (Verma *et al.*, 2013, 2014; Verma *et al.*, 2015; Verma *et al.*, 2016a);

Arthrobacter, *Bacillus* and *Bacillus* derived genera *Pseudomonas* as phosphate, potassium and zinc solubilizing bacteria (Verma *et al.,* 2016b; Yadav *et al.,* 2016) and *Trichoderma* and *Arbuscular mycorrhiza* as carbon mineralizing fungi (Brahmaprakash and Sahu 2012). Bio-inoculants contain beneficial microbes that enhances plant growth, when applied to the field, by the asset of its nutrient solubilization (Amalraj *et al.,* 2012; Yadav *et al.,* 2015a), nitrogen fixation (Sivasakthivelan and Saranraj 2013; Suman *et al.,* 2008; Yadav *et al.,* 2015b; Suman *et al.,* 2015), phytohormone production (Bottini *et al.,* 2004) and induction of defense mechanism (Richardson *et al.,* 2009) resulting in also improved soil health and productivity.

Carrier based biofertilizers has already proved to be best over the agro-chemicals and have been showing the tremendous effect on the global agriculture productivity since past few decades. Bhattacharyya and Kumar (2000), stated that bioinoculants manufactured in India are mostly solid carrier based and have a shelf life of only six months.

These carrier based inoculants are inherent with certain constrains like lower shelf-life, poor survival under adverse environmental conditions, high degree of contamination, and inconsistence field performances. There have been many attempts to find alternatives for carrier based inoculants and also to enhance viability of microorganisms in the bioinoculants.

In future agriculture, chemical fertilizers desires would result in further defeat in soil health, feasibilities of water pollution and calculated load on the fiscal system (Davidson *et al.,* 2013). Haphazard synthetic fertilizer usage has polluted the soil, water basins, destroyed micro-organisms and eco-friendly insects, made the crop more susceptible to diseases and depleted soil fertility. In this critical context microorganisms have been emerged as the potential alternative for the productivity, reliability and sustainability of the global food chain.

Hydrogel is a particular class of macromolecular gels, obtained by chemical stabilization of hydrophilic polymers in a three-dimensional network, in which the dispersed phase is water, present in substantial quantity (Narjary *et al.,* 2012). Currently, superabsorbent hydrogel are widely used as absorbent core materials (Buchholz and Peppas 1994). Degiorgi *et al.,* (2002), investigated that the activity of microorganisms and mycorrhiza will increase the using super absorbents. Super absorbents have 100 % natural structures and don't have any harm to the environment. Many studies related to application of hydrogel in horticulture have been reported (Henderson and Hensley 1985; Ingram and Yeager 1987; Wang and Boogher 1987; Wang and Gregg 1990). It was observed that the application of hydrogel to sandy soils improved water availability to plants by increasing the retention pores and reduced saturated hydraulic conductivity by decreasing the drainage pores.

Rectifying the disadvantages of the liquid and lignite carrier based biofertilizers, hydrogel based biofertilizers have been developed which would be the only alternative for the cost effective sustainable agriculture.

Hydrogel based bioinoculants increase the shelf life of bioinoculants to reduce crises such as soil erosion, frequent droughts or providing food security requires knowledge of their behaviors and performances in the soil.

Materials and Methods

Microorganisms, Growth Medium and Carriers

Selection of effective microbes for developing microbial consortia, based on efficient plant growth promoting traits, i.e. *Azotobacter chroococcum* for nitrogen fixation, *Pseudomonas fluorescence* for phosphate solubilization and *Trichoderma viride* for phyto-stimulation. These microbes have been identified using 16S rRNA and ITS gene sequencing and BLASTn analysis as described by Yadav (Yadav *et al.*, 2014b; Yadav *et al.*, 2014a).

These microbes have been screened for different plant growth promoting attributes (Verma *et al.*, 2013, 2014). The tryptone yeast extract agar (g L^{-1}: tryptone: 20.0, yeast extract: 10.0, mannitol: 10.0, agar: 20); King's B agar (g L^{-1}: 20 protease peptone; 1.5 K_2HPO_4; 1.5 $MgSO_4.7H_2O$; 10 ml glycerol; 18 agar; pH 3-6±0.2) and potato dextrose agar (g L^{-1}: 4 potato infusion (from 200 g potato), 20 dextrose, supplemented with 50 µg/mL chloramphenicol; 20 g agar) were used to culture *Azotobacter chroococcum*, *Pseudomonas fluorescence* respectively and *Trichoderma viride*.

These microbial cultures were also morphologically characterized and details of cultures. An indigenous and novel superabsorbent material of semi synthetic chemical nature was developed and evaluated for its characteristics and potential.

This product has been christened 'Pusa Hydrogel' by the Indian Agricultural Research Institute. Pusa hydrogel is natural polymer absorbs water 400 times it's dry weight and gradually releases the same. It significantly improves physical properties of soils and fertility (Table 1).

Microbial Compatibility of Microbes and Concentration of Hydrogel for Microbial Growth

Selected plant growth promoting microbial cultures were tested for their compatibility under *in vitro* condition by co-culturing on different growth media. Experiment was set up for bacterial × bacterial and bacterial × fungal interaction studies. The isolates showing compatibility were further used for making microbial consortium. Screening of different concentrations of hydrogel ranging from 0.5 to 6 %, Gel was prepared in double distilled water and sterilized. The characteristics of the each gel preparation were recorded after it was cooled to room temperature. The 0.5 and 1.0 % gel was found to be liquid even after cooling, 2-4 % was found to be semi solid, but 5 and 6 % solidified at room temperature after air drying. The optimum population of microbes in the given media is very important to make use of it for further beneficial purpose. Hence to know the log phase of the given microbial cultures, growth kinetics of each bacteria/fungi was studied under *in vitro* conditions. The known population of microbial culture was inoculated to 50 mL culture flasks containing 0, 0.5, 1, 2 and 3 % gel in the respective sterilized growth medium. These are being incubated at 30 °C for the growth kinetics. The observations recorded at regular intervals on CFU mL^{-1}, zero, 7^{th}, 15^{th} days and 1^{st}, 2^{nd}, 3^{rd} month after incubation, samples were drawn, serially diluted and plated for the viable cell count.

Shelf Life and Bio-Efficacy Studies of Carrier Based Bioinoculants

Bioinoculants (*Azotobacter chroococcum*, *Pseudomonas fluorescence* and *Trichoderma viride*) shelf life studies with three carriers' lignite, liquid and hydrogel were prepared with the cell population

adjusted to 5×10^{10} CFU mL^{-1} and made upto 250 mL were packed in surface sterilized high density polythene (HDPE). The formulated products were stored in culture bottles at room temperature and assessed for their shelf life, pH and temperature at monthly interval upto 36 months. Bio-efficacy of bioinoculants was studied by pot culture assay with wheat as the test crop. Twenty seven treatments in triplicate were three microbial formulations, *viz*. *Azotobacter chroococcum* (AC), *Pseudomonas fluorescence* (PF), *Trichoderma viride* (TV) and AC+TV, AC+PF, PF+TV, AC+ PF+ TV and control with three carriers lignite, liquid and hydrogel. Soil and sand was mixed in 3:1 proportion and sterilized at 121°C for 1 h consecutively for three days. Each plastic bag (10×12 inches) was filled with five kg soil. Wheat (*var*. HD2951) seeds were surface sterilized and treated with bioinoculants in different combination (Table 2) @ 10 mL kg^{-1} seed in case of liquid inoculants or 5 mL of each inoculant for consortia treatments. In each pot seeds were sown at 2 cm depth and at equal distance between the seeds. Six replications were maintained for all the treatments. In each pot exactly 6 plantlets were maintained by thinning the unevenly grown plant at 15 day after sowing (DAS). The wheat plants were uprooted carefully on 45^{th} and 90^{th} DAS and analysed for various agronomical parameters like root, shoot length, fresh and dry weight and chlorophyll.

Results and Discussion

Characterization of Hydrogel

Hydrogel has special trait of high water absorption, non-toxicity to microbial cells and stability in soil for as a carrier matrix. It absorbs water 400 times and releases gradually and thus helps in overcoming moisture stress (Table 1). Among seven different hydrogel concentration 0.5, 1, 2, 3, 4, 5 and 6 % gel at 0.5 and 1.0 % found to be liquid even after cooling, 2-4 % was found to be semi solid, but 5 and 6 % solidified at room temperature after air drying. 0.5 to 3 % gel was found to be effective concentration for growth of microbial inoculants (Fig. 1).

Evaluation of Viability of Microbial Inoculants in Hydrogel

The growth survival of hydrogel based microbial inoculants are continuing but the observations for the period 0 to 30 days clearly indicate that addition of gel in the growth medium provided better condition for the growth of both bacteria and fungi. Control of each culture in isolation has been included in the experiment for deciphering the anti-culture effect on each other in the microbial consortium. The population of bacteria inoculated to 0.5, 1, 2 and 3 % hydrogel based medium was shown increase in population with advance in incubation time. The highest population was observed in 2 % hydrogel concentration. A combination of *Azotobacter chroococcum*, *Pseudomonas fluorescence* and *Trichoderma viride* has been used for developing gel based microbial consortium utilizing 2 % gel strength. The hydrogel based formulation of *Azotobacter chroococcum*, *Pseudomonas fluorescence* and *Trichoderma viride* showed 1.6×10^{6}, 2.3×10^{5} and 4.6×10^{3} CFU mL^{-1} respectively after three month incubation (Fig. 2). The increase in population of microbes was observed at 7 day after initial (DAI) while, the population was become steepen/studies in general upto two years.

Survivability and Shelf life Studies of Carrier Based Bio-inoculants

Bioinoculants shelf life studies with three carriers, after 90 days of incubations

Azotobacter chroococcum exhibited 1.2×10^7, 1.4×10^8 and 3.5×10^9 CFU mL^{-1}, *Pseudomonas fluorescence* 2.2×10^7, 2.4×10^8 and 4.5×10^9 CFU mL^{-1} and *Trichoderma viride* 1.4×10^6, 2.8×10^7 and 2.5×10^8 CFU mL^{-1} with three carriers lignite, liquid and hydrogel respectively. The observations for the period up to 90 days clearly indicated that addition of gel in the growth medium provided better condition for the growth of both bacteria and fungi compared to liquid and lignite conditions. In all the cases, the initial set population 5×10^{10} CFU mL^{-1} significantly increased with the time. However, the degree of declination varied with the type of microbial inoculants. The survival of three inoculants formulation was recorded upto 90 days under room temperature. The hydrogel based bioinoculants *viz*. *Azotobacter chroococcum*, *Pseudomonas fluorescence* and *Trichoderma viride* showed maximum population compare to liquid and lignite 9.6×10^8, 3.9×10^7 and 2.9×10^7 CFU mL^{-1} at 90 days respectively. The mean temperature and pH of all the formulations were recorded at monthly interval.

Evaluation of Hydrogel Based Bio-inoculants

Among eight different treatments, wheat seeds treated with consortium of AZ+PF+TV and AZ+PF showed significantly growth promotion by the virtue of increased root, shoot length, fresh, dry weight and chlorophyll as compared to other treatments. Plant growth parameters were recorded on wheat plant upon inoculation of microbial based formulations compared to control both at 45 and 90 DAS (Fig. 3). Highest mean shoot and root length was recorded with hydrogel based carrier at 45 (32.9 and 5.7 cm) and 90 DAS (50.1 and 7.9 cm). The highest fresh and dry weight was recorded with hydrogel based formulation at 45 (1.6 and 0.3 g) and 90 DAS (3.4 and 1.9 g), along with microbial treatments, the *Pseudomonas fluorescence* treatment was found to be best as indicated (Fig. 3b). The variation in the population of microbes in the rhizospheric soil of wheat was recorded at 45 and 90 DAS (Fig. 3).

Table.1 Characteristics and Potential Applications of Hydrogel

SN	Parameter	Characteristic and potential applications
1.	Chemical constitution	Cross linked anionic polyacrylate
2.	Appearance	Amorphous granules
3.	Particle size	20-100 mesh (micro granules)
4.	pH	7.0-7.5
5.	Stability at 50°C	Stable
6.	Sensitivity of UV light	Not sensitive
7.	Temperature	40-50 °C
8.	Stability	~ 2 Years
9.	Other properties	• Absorbs water 400 times it's dry weight and gradually releases the same. • Exhibits maximum absorbency @ temperatures (40-50 °C) characteristic of semi-arid and arid soils • Improves % seed germination and seedling emergence rate • Reduces the irrigation and fertigation requirements and dose of urea to be applied

Table.2 Treatments used to Evaluate the Plant Growth Promoting Effect of Bio-Inoculants

Treatment	Different microbial combinations
T1	*Azotobacter chrococcum*
T2	*Pseudomonas fluorescence*
T3	*Trichoderma viride*
T4	*Azotobacter chrococcum* + *Trichoderma viride*
T5	*Azotobacter chrococcum* + *Pseudomonas fluorescence*
T6	*Pseudomonas fluorescence* + *Trichoderma viride*
T7	*Azotobacter chrococcum* + *Pseudomonas fluorescence* + *Trichoderma viride*
T8	Control (without inoculation)

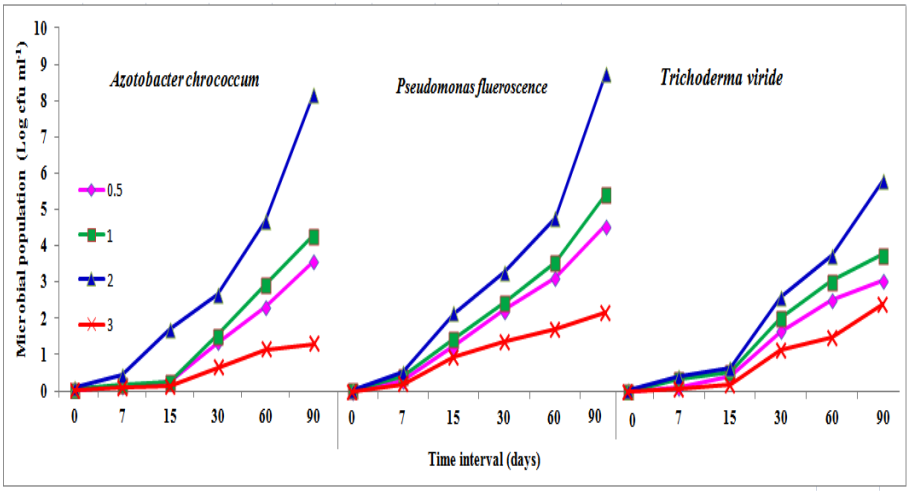

Fig.1 Survivability of Microbial Bioinoculants *Azotobacter Chroococcum*, *Pseudomonas fluorescence* and *Trichoderma Viride* in 0.5, 1, 2 and 3 % Hydrogel

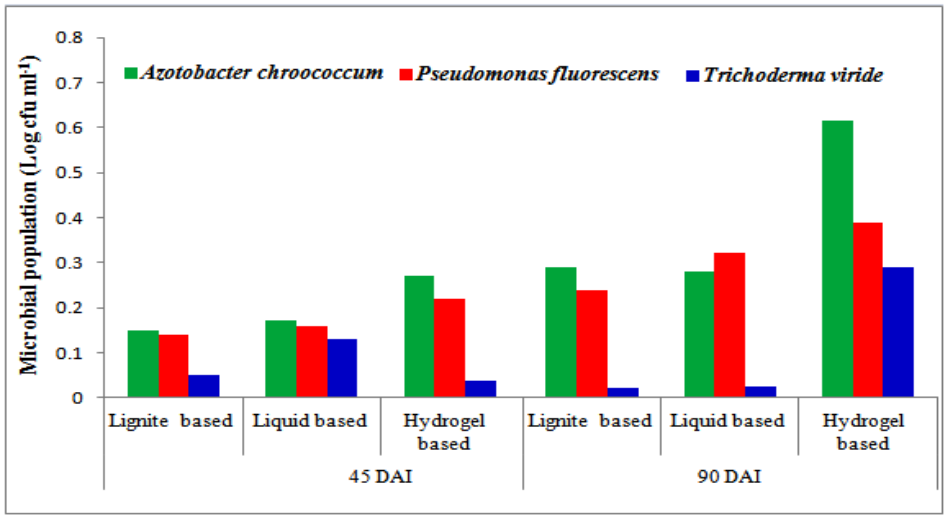

Fig.2 Shelf Life Studies of Carrier based Bio-inoculants *Azotobacter chroococcum*, *Pseudomonas fluorescence* and *Trichoderma viride* in Lignite, Liquid and Hydrogel. DAI-Day after Inoculation

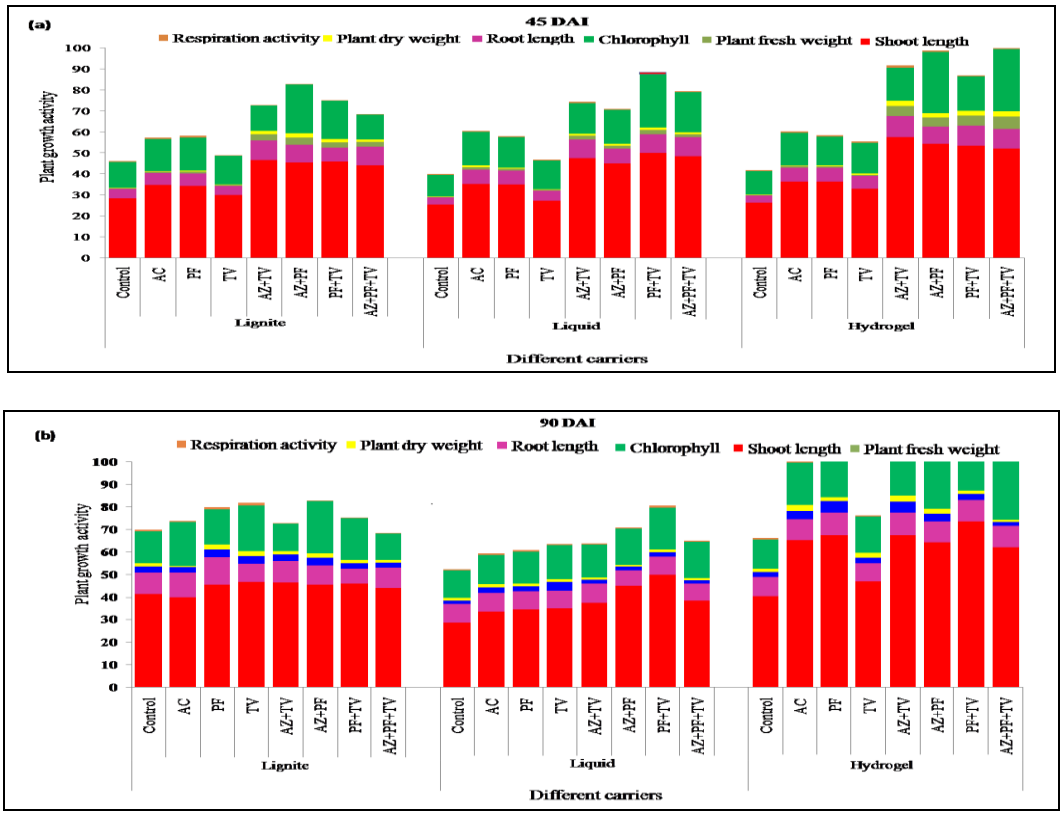

Fig.3 Evaluation of Hydrogel Based Bio-Inoculants (A) 45 Dai (B) 90 Dai (Day After Inoculation) Ac: *Azotobacter chroococcum* Pf: *Pseudomonas fluorescence* and Tv: *Trichoderma viride*

Overall results indicate that the hydrogel based formulations of *Pseudomonas fluorescence* is found to be of high potential in improving wheat plant growth compared to liquid and lignite based formulations. Microbial combination were showed highest mean shoot and root length was recorded with hydrogel based carrier at 45 (35.6 and 7.3 cm/ plant) and 90 DAS (51 and 8.8 cm/plant). The highest fresh and dry weight (g/plant) was recorded with hydrogel based formulation at 45 (0.9 and 0.4 g/plant) and 90 DAS (4.14 and 2.18 g/plant). Among microbial consortia treatments, *Azotobacter chroococcum* + *Pseudomonas fluorescence* + *Trichoderma viride* treatment was found to be best. The difference in the population of microbes in the rhizosphere soil of wheat was recorded at 45 and 90 DAS. Overall results indicate that the hydrogel based formulations of *Azotobacter chroococcum* + *Pseudomonas fluorescence* + *Trichoderma viride* is found to be of high potential in improving wheat plant growth compared to liquid and lignite based formulations.

The successful establishment of agricultural crops depends on moisture availability and is often restricted by poor soil moisture level particularly in arid and semi-arid environments. The use of soil conditioners has been suggested to improve moisture retention by coarse soils (McGuire *et al.*, 1978). The gel used in the present study had high water absorption during the wetting and it increased survivability of microbial cultures for longer shelf life. The chemicals and ions present in irrigation water can adversely affect the water retention by hydrogel (Johnson 1984; Asady *et al.*, 1985). Water retention was significantly increased with hydrogel for irrigation

compared to liquid and lignite.

Shelf life is the first and foremost problem of the carrier based biofertilizers which is up to 3 months and it does not retain throughout the crop cycle, liquid biofertilizers on the other hand facilitates the long survival of the organism by providing the suitable medium which is sufficient for the entire crop cycle. Moisture retaining capacity of the carrier based biofertilizers is very low which does not allow the organism viable for longer period and the liquid biofertilizers facilitates the enhanced viability of the organism (Mahdi *et al.,* 2010) but not moisture retention. The hydrogel based formulation of *Azotobacter*, *Pseudomonas* and *Trichoderma* showed instantly increase population upto two years as compare to obtained results, while increasing in moisture retention in light soils, polymers can address permeability problems in heavy soil and problems in leaching fertilizer and microorganisms viable for longer period.

Azotobacter belongs to the genus diazotrophic bacteria which is a free-living organism whose resting stage is a cyst. It is abundantly found in neutral to alkaline soils, in aquatic environments, and on some plants. *Azotobacter* is capable of performing several metabolic activities, including atmospheric nitrogen fixation by conversion to ammonia. *Azotobacter* sp. has the highest metabolic rate of any organisms. It serves as potential biofertilizers for all non-leguminous plants especially rice, cotton, vegetables etc. *Azotobacter* population is high in rhizosphere region. The lack of organic matter in the soil is a limiting factor in the proliferation of *Azotobacter* in the soil. Inoculants *Pseudomonas* of phosphate solubilizing bacteria as fertilizer increases P uptake by the plant and enhance crop yield. There are many strains of different genera which promote the phosphate solubilization among which; strains from the genera *Pseudomonas* are the most powerful phosphate solubilizers. Phosphate solubilization by the organisms mainly involves the production of organic acids which is the principle mechanism solubilization, and the organic phosphorous in the soil is mineralized by acid phosphates which is a vital step.

The plant growth-promoting efficacy of *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*, re-suspended in either sterile distilled water or phosphate saline buffer (cell suspension) has already been demonstrated in different agriculture crops under greenhouse conditions (Luna *et al.,* 2010; Muthukumarasamy *et al.,* 2005; Suman *et al.,* 2005; James *et al.,* 2002; Mehnaz and Lazarovits 2006). However, the use of without carrier based bio-inoculants and liquid formulations have not been reported earlier.

The development of hydrogel bioinoculants assumes greater importance in sustainable crop protection which could increase the shelf life providing tolerance to increase adverse conditions. Three microbial isolates *Azotobacter chroococcum, Pseudomonas fluorescence* and *Trichoderma viride* are already reported with effective plant growth promoting properties such as ability to fix nitrogen, solubilization of PO_4 and antifungal activity were selected for hydrogel based microbial formulation and tested for their effect on the growth of wheat by pot assay.

The results of our studies showed that the locally prepared hydrogel has reasonably high water absorption capacity that was retained in subsequent wetting and drying cycles. The addition of the hydrogel improved the water storage of soils and enhanced seedling growth (Akhter *et al.,*

2004). The use of hydrogel amendments as cultural practice will be useful for increased plant establishment in drought prone environments. However, the variations in the influence of hydrogel addition on soil moisture retention properties and in the response of different species warrant further investigations for the recommendations of specific hydrogel and soil types. Nevertheless, the hydrogel used in this work has proven to have water absorption comparable to commercially sold products.

Preservation medium and storage temperature strongly influenced the viability of both *Acetobacter diazotrophicus* L1 and *Herbaspirillum seropedicae* J24 in liquid formulations. Storage of liquid formulations at 25°C is not recommended because cell viability decreases rapidly in 2-3 months. In contrast, some of the liquid formulations stored at 4°C maintained their viability for several months (Pindi 2012). Oxidative stress may be one of the important factors leading to decrease in viability of microbial cells in liquid formulation (Jakubowski *et al.,* 2000; Patiño-Vera *et al.,* 2005). In the present study, viability of *Azotobacter chroococcum, Pseudomonas fluorescence* and *Trichoderma viride* in microbial formulation was significantly improved by applying hydrogel as protectant and enhanced shelf life by combining as consortia.

Impermeable hydrogel biodegradation in soil was increased bulk density of soil. As an effective material, super absorbent polymer were identified as effective in reducing the effects of drought stress and thereby increasing plant resistance to stresses and increasing plant performance. Super absorbent polymers cause to increase aggregate stability and prevent crust formation, prevent on farm runoff formation and reduce soil erosion. And the most important benefit of hydrogel usage is preventing deep penetration of water of root environment and leaching salts and its effect on the accumulation of proline and soluble sugars. Future studies on optimization shelf life of microbial formulation with hydrogel upto two years and application of these formulations on different field crops are in enhances to exploit the plant growth promotion and soil health for agriculture exaltation.

In conclusion, development of low-cost and higher survivability of hydrogel biofertilizers was conducted at Agricultural chemical, Indian Agricultural Research Institute (India). Three biofertilizers inoculums were developed into microbial formulations of hydrogel based biofertilizers. Tryptone yeast extract mannitol agar hydrogel based biofertilizers kept at room temperatures showed significantly high survival rates after storage for up to two years as compared to other formulations and treatments. The hydrogel based biofertilizers have great potential to be promoted as low-cost and long shelf-life products suitable for soilless and other innovation cultivation system in the Indian agriculture.

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Ethical Standard

The experiments undertaken comply with the current laws of India, the country where

the investigation was undertaken.

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