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In vitro Studies on the Effects of Biofertilizers (*Azotobacter* and *Rhizobium*) on Seed Germination and Development of *Trigonella foenum-graecum* L. using a Novel Glass Marble containing Liquid Medium

G.S. Nagananda, Arijit Das, Sourav Bhattacharya and T. Kalpana
Department of Plant Biotechnology, Genohelix Biolabs, Jain University, 127/3, Bull Temple Road, Chamarajpet, Bangalore-560019, Karnataka, India

Abstract: Biofertilizers are the formulations of living microorganisms, which are capable of fixing atmospheric nitrogen in the soil and thereby, increasing the crop yield. *Trigonella foenum-graecum* L. is a medicinally important plant possessing anti-diabetic, anti-cancerous, anti-microbial and hypocholesterolaemic properties. The present study was conducted to develop an *in vitro* method for studying the effects of biofertilizers (*Azotobacter* and *Rhizobium*) on the seed germination and development of *Trigonella foenum graecum* L. using a simple and cost-effective liquid culture medium containing glass marbles as reusable and biologically inert support matrix. Sucrose optimization studies revealed maximum development for the plantlets grown on 1X Murashige and Skoog liquid medium containing 4% sucrose and glass marbles. *Azotobacter* and *Rhizobium* were isolated from rhizosphere soil and root nodules of *Trigonella* plants, respectively and identified following the standard procedures. Mass cultivation of the bacteria carried out for 5 days reported counts of 2.3×10^4 cells mL^{-1}. The harvested bacterial cells were used to coat the seeds in the presence and absence of charcoal. After 15 days of growth under *in vitro* conditions, the root length, shoot length, fresh weight, protein, carbohydrate and chlorophyll contents of the plantlets were determined. Maximum growth was observed for the plantlets grown on 1X MS medium with 4% sucrose and glass marbles, inoculated with 40% concentration of *Azotobacter*, *Rhizobium* and their co-inoculum mixed with charcoal. Field trials, conducted under green house conditions, revealed that 10% biofertilizer co-inoculum supported maximum growth of the plants when the seeds were coated with charcoal.

Key words: *Trigonella foenum- graecum* L., biofertilizers, charcoal, glass marble, Murashige and Skoog medium

INTRODUCTION

Chemical fertilizers pose a health hazard and affect the microbial population in soil by degrading the physical structure of the soil, leading to a lack of oxygen in the plants root zone besides being quite expensive and making the cost of production high. In such a situation the role of biofertilizers may be explored as an alternative for enhancing the soil fertility. Biofertilizers are the formulations of living microorganisms, which are able to fix atmospheric nitrogen in the available form to plants, either by living freely in the soil or being associated symbiotically with plants (Chandrasekar *et al*., 2005). They are capable of mobilizing nutritive elements from non-usable form to usable form through biological processes (Tien *et al*., 1979). Biological nitrogen fixation is carried out by both symbiotic and free living bacteria and blue green algae. Symbiotic nitrogen fixation provides 80% of the biologically fixed nitrogen on land. Nitrogen fixing bacteria are very selective in choosing roots of particular legumes species to infect, invade and form root nodules (Chandrasekar *et al*., 2005).

Rhizobium has the exceptional ability to form nodules on roots or stems of leguminous plants. Free living diazotrophs promote the rhizobial efficiency by altering root architecture providing more niches for nodulation and thus enhance the nitrogen-fixing ability of legumes. Legume inoculation with *Rhizobium* is an aged practice that has been carried out for more than a century in agricultural systems (Qureshi *et al*., 2009). It is a promising fertilizer because it is easy to handle and improves the plant growth and seed quality. *Azotobacter*, a free-living diazotroph has also been reported to produce beneficial effects on crop yields through a variety of mechanisms including biosynthesis of biologically active substances, stimulation of rhizospheric

Corresponding Author: G.S. Nagananda, Department of Plant Biotechnology, Genohelix Biolabs, Jain University, 127/3, Bull Temple Road, Chamarajpet, Bangalore- 560019, Karnataka, India Tel: +919844485920

microbes, modification of nutrient uptake and ultimately boosting biological nitrogen fixation (Somers *et al*., 2004). Its use in agricultural practices can increase the crop yields, being economical and environmental friendly at the same time. Co-inoculation of legumes with symbiotic and free living microbes like *Azotobacter*, *Azospirillum* and *Acetobacter* has received great attention in recent years (Dashti *et al*., 1998).

The large scale commercial propagation of crop yields is highly based on plant tissue culture techniques. Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment (Ahloowalia *et al*., 2004). Different techniques in plant tissue culture include seed culture, embryo culture, shoot tip culture, apical meristem culture, nodal culture, root culture, nucellus and endosperm culture. By using the techniques of plant tissue culture *in vitro* studies on various aspects like biofertilizers, development of resistant varieties, study of developmental patterns, etc., can be carried out. *In vitro* culturing of seeds can also be a tool to obtain disease-free plants and also to know the nutritional requirement for the growth and development of plants.

In the search for an alternative, eco-friendly and economical constituent of tissue culture media, a number of support matrices (Kong and Chin, 1988; Henderson and Kinnersley, 1988; Bhattacharya *et al*., 1994; Babbar and Jain, 1998; Babbar *et al*., 2005, Jain and Babbar, 2006) have been tested as potent alternatives to agar. The National Research Development Corporation, India has listed cotton fibers, glass wool, nylon cloth, glass beads, filter paper, etc. as low cost agar alternatives (NRDC, 2002) but most of these materials are less explored in application than agar and have their own limitations.

In this present study, the effects of biofertilizers like *Azotobacter* and *Rhizobium* on seed germination and development of *Trigonella foenum graecum* L. under *in vitro* conditions were investigated on a novel, cost-effective, glass marble containing liquid medium.

MATERIALS AND METHODS

The present study was conducted during the period from 03.12.2009 to 14.03.2010 at Genohelix Biolabs, Jain University, Chamarajpet, Bangalore, Karnataka, India.

The chemicals which were used in the present study are of A.R grade chemicals from Nice, sd fine, Qualigens, Hi-media, Spectrochem, Colloids and Loba chemie.

Sterilization of explant: *Trigonella foenum-graecum* L. seeds, purchased from the local market, were used as the explant. The seeds were washed thoroughly under tap water, surface sterilized using liquid detergent 2% (v/v) Savlon, 6-8 drops of Tween-20 for 15 min, rinsed with 70% ethanol for 30 sec, disinfected with 0.05% (w/v) $HgCl_2$ for 6 min and again rinsed in sterile water several times to remove the traces of $HgCl_2$.

Standardization of sucrose: The sterilized seeds were cultured on 1X MS (Murashige and Skoog, 1962) medium containing different concentrations of sucrose ranging from 0 to 4% with glass marbles (1 cm diameter) as a supporting source. Five seeds were inoculated in each culture bottle. The cultures were maintained in the culture room at a temperature of 25±2°C, light intensity of 1000 LUX, relative humidity between 50-60%, under photo-periodic regime for 16 h light and 8 h dark cycle. The data were collected after 15 days of incubation; phenotypical traits and physiological estimations were carried out.

Isolation of rhizobia from root nodules of *Trigonella foenum-graecum* L.: One gram of root nodules was collected from *Trigonella foenum-graecum* L. roots. The nodules were washed thoroughly under tap water, surface sterilized using liquid detergent 2% (v/v) Savlon, 6-8 drops of Tween-20 for 15 min, rinsed with 70% ethanol for 30 sec, disinfected with 0.05% (w/v) $HgCl_2$ for 6 min and again rinsed in sterile water several times to remove the traces of $HgCl_2$. The nodules were crushed with a sterile glass rod and the suspension was serially diluted till 10^{-5} dilution. 0.1 mL of each dilution was plated on Yeast Extract Mannitol Agar (YEMA) using spread plate technique. The plates were incubated at room temperature for 4-7 days.

Isolation of *Azotobacter*: One gram of soil was aseptically collected from the Institute campus and serially diluted till 10^{-5} dilution. 0.1 mL of each dilution was plated on Ashby's agar using spread plate technique. The plates were incubated at room temperature for 4-7 days.

Following incubation, the isolated colonies were pure cultured and Gram stained. Biochemical characterization of the isolated colonies was carried out using standard protocols (Kannan, 2002). Identification was carried out according to Bergey's Manual (7th Edn.).

Mass production of *Azotobacter* and *Rhizobium*: Five hundred milliliter of Jensen's broth was prepared containing (g L^{-1}): K_2HPO_4, 1; $MgSO_4$, 0.5; NaCl, 0.5; $FeSO_4$, 0.100; $NaMoO_4$, 0.005; $CaCO_3$, 2; Sucrose, 20 ; pH

7.0 and 500 mL of Yeast Extract Mannitol broth was prepared containing (g L^{-1}): mannitol, 10; $CaCO_3$, 4; K_2HPO_4, 0.5; $MgSO_4$, 0.2; yeast extract, 0.4; NaCl, 0.1, pH 7.0 and sterilized. The media were separately inoculated with pure culture of *Azotobacter* and *Rhizobium*, respectively and incubated in shaker incubator at 30°C for 3 to 5 days.

Cell harvesting: The broth cultures were centrifuged at 5000 rpm for 30 min at 4°C, the pellets were resuspended in physiological saline and stored at 4°C.

Coating of *Trigonella foenum-graecum* L. seeds with different concentrations of *Azotobacter* and *Rhizobium*: Two sets of 25 mL each Jensen's broth with 3% (w/v) sucrose and YEM broth with 3% (w/v) sucrose were prepared and sterilized. Five hundred microliter of physiological saline suspension of *Azotobacter* and *Rhizobium* were aseptically added to the sterile broths and different concentrations (10, 20, 30 and 40%) of *Azotobacter* and *Rhizobium* inocula were separately prepared in respective culture broth and were added to the sterile culture bottles containing 1 g of activated charcoal and another set without activated charcoal. One hundred gram of surface sterilized seeds were separately mixed with each inoculum and kept for overnight incubation.

Inoculation of *Trigonella foenum graecum* L. seeds coated with *Azotobacter* and *Rhizobium* inocula: The seeds coated with different concentrations (10-40%) of *Azotobacter* and *Rhizobium* inocula (with and without charcoal) were cultured on 1X MS medium with 4% sucrose and glass marbles as supporting source and incubated under controlled conditions. Seed germination and plant growth were regularly monitored and after 15 days of incubation phenotypic traits and physiological estimations were carried out.

Coating of *Trigonella foenum graecum* L. seeds with co-inoculum of *Azotobacter* and *Rhizobium* : Two sets of 25 mL each Jensen's broth with 3% (w/v) sucrose and YEM broth with 3% (w/v) sucrose were prepared and sterilized. Five hundred microliter of physiological saline suspension of *Azotobacter* and *Rhizobium* were aseptically added to the sterile broths. Another set of 25 mL of sterile medium was prepared containing 1:1 (v/v) Jensen's broth with 3% (w/v) sucrose and YEM broth with 3% (w/v) sucrose. Forty percent concentration of each inoculum which gave the best result in the previous step was added to this medium. Different concentrations (10, 20, 30 and 40%) of this co-inoculum were separately prepared in 1:1 respective broth mixture and were added to the sterile culture bottles containing 1 g of activated charcoal and another set without activated charcoal. Hundred gram of surface sterilized seeds were separately mixed with each concentration of co-inoculum and kept for overnight incubation.

Inoculation of seeds coated with co-inoculum of *Azotobacter* and *Rhizobium* under *in vitro* condition: The seeds coated with different concentrations (10-40%) of *Azotobacter* and *Rhizobium* co-inocula (with and without charcoal) were cultured on 1X MS medium with 4% sucrose and glass marbles as supporting source and incubated under controlled conditions. Seed germination and plant growth were regularly monitored and after 15 days of incubation phenotypic traits and physiological estimations were carried out.

Field study of seeds coated with co-inoculum of *Azotobacter* and *Rhizobium*: Soil was collected from the agricultural field, sterilized at 121°C for 15 min at 15 lbs pressure. The sterile soil was cooled to room temperature and dispensed into the sterile pots and watered. The seeds coated with different concentrations of (10-40%) co-inoculum of *Azotobacter* and *Rhizobium* (with and without charcoal) were sowed in the pots and were maintained in green house. After 15 days phenotypical traits and physiological estimations were carried out as per standard protocols.

Estimation of protein: One gram of plant material was weighed, ground with 5 mL of 0.2 M phosphate buffer (pH 7) and filtered using Whatman's No. 1 filter paper. The filtrates were subjected to protein estimation by Lowry's method (Lowry *et al*., 1951) with absorbance measured at 660 nm.

Estimation of total carbohydrate: One hundred milligram of plant material was hydrolyzed with 2.5 N HCl by incubating in boiling water bath for 15 min and filtered using Whatman's No. 1 filter paper. The filtrates were estimated for total carbohydrate content by Anthrone method (Hedge and Hofreiter, 1962) with absorbance measured at 630 nm.

Estimation of chlorophyll: One gram of plant material was weighed and ground with 5 mL of 80% acetone and centrifuged at 5000 rpm for 5 min and the supernatant was transferred to a 10 mL volumetric flask. The volume was made upto 10 mL using 80% acetone and the absorbance of the solution was measured at 645, 652 and 663 nm, respectively (Sadasivam and Manickam, 1997).

RESULTS

In the present investigation, the influence of different concentrations of sucrose on the germination of seeds and development of plants were studied. 100% of seed germination was observed on 2nd day of inoculation for all the concentrations of sucrose used. After 15 days of incubation, the results of phenotypic traits showed the maximum shoot length (4.60±0.42 cm), root length (3.74±1.00 cm) and fresh weight (0.17±0.03 g) for the plantlets grown on 1X MS medium with 4% sucrose and glass marbles (Fig. 1a-h). The physiological estimations also supported the phenotypic traits results with maximum protein (1.67±0.01 mg mL^{-1}), carbohydrate (0.015±0.00 mg mL^{-1}) and chlorophyll content (0.06±0.01 mg g^{-1}) (Table 1).

For *in vitro* studies on the effects of biofertilizers on germination and development of *Trigonella foenum-graecum* L., various nitrogen-fixing bacteria like *Azotobacter* and *Rhizobium* were isolated from the rhizosphere soil and root nodules of *Trigonella foenum-graecum* L., respectively. Gram's staining

Fig. 1: (a-c): *In vitro* seed germination and development of *Trigonella foenum-graecum* L. on 1X MS medium with 4% sucrose containing glass marble as a supporting matrix. (d-h) Phenotypic trait results of sucrose standardization (0-4%). (i) Seeds coated with charcoal and (j) seeds coated without charcoal

Table 1: Effect of different concentrations of sucrose on *in vitro* seed germination and development of *Trigonella foenum-graecum* L. on glass marble containing MS medium

	Phenotypical traits			Physiological estimations		
Conc. of sucrose (%)	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Protein (mg mL^{-1})	Carbohydrate (mg mL^{-1})	Total chlorophyll (mg g^{-1})
0	3.22±0.58	2.18±0.98	0.14±0.02	1.11±0.00	0.005±0.00	0.03±0.00
1	3.64±0.31	2.40±1.20	0.15±0.03	1.19±0.00	0.009±0.00	0.04±0.00
2	4.12±0.64	2.52±0.92	0.15±0.03	1.22±0.00	0.010±0.00	0.04±0.00
3	4.50±0.39	3.60±1.10	0.16±0.02	1.57±0.01	0.010±0.02	0.05±0.01
4	4.60±0.42	3.74±1.00	0.17±0.03	1.67±0.01	0.015±0.00	0.06±0.01

Table 2: Partial biochemical characterization of *Azotobacter* and *Rhizobium* isolates

Biochemical tests	*Azotobacter*	*Rhizobium*
Colony texture	Mucoid	Mucoid
Cell shape	Cocco-bacilli	Pleomorphic rods
Gram's reaction	Negative	Negative
Indole production	+	–
Methyl red	+	–
Voges proskauer	–	–
Citrate utilization	+	–
Urease	+	–
Motility	–	–
Sugar fermentation		
Glucose	+	+
Mannitol	+	+

Table 3: Effects of different concentrations of *Azotobacter* and *Rhizobium* inocula on *in vitro* seed germination and development of *Trigonella foenum-graecum* L. on glass marble containing 1X MS medium with 4% (w/v) sucrose

		Phenotypical traits			Physiological estimations		
Inoculum used for coating	Conc. of inoculum (%)	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Protein (mg mL^{-1})	Carbohydrate (mg mL^{-1})	Total chlorophyll (mg g^{-1})
Azotobacter	10	3.60±0.99	3.20±0.32	0.16±0.04	0.36±0.07	0.14±0.02	0.06±0.01
	20	4.10±0.64	3.40±0.64	0.16±0.05	0.40±0.07	0.23±0.04	0.06±0.01
	30	4.00±0.37	3.60±1.11	0.17±0.01	0.40±0.09	0.26±0.05	0.06±0.01
	40	4.40±0.39	3.90±0.62	0.17±0.08	0.66±0.13	0.29±0.05	0.14±0.02
Azotobacter with charcoal	10	3.80±1.13	5.16±0.51	0.12±0.01	0.61±0.12	0.16±0.03	0.18±0.01
	20	4.18±0.37	5.76±1.14	0.16±0.03	0.62±0.12	0.33±0.06	0.18±0.01
	30	4.52±0.97	6.38±1.49	0.16±0.05	0.65±0.13	0.42±0.08	0.19±0.01
	40	5.16±0.53	7.60±0.98	0.18±0.04	0.88±0.17	0.47±0.09	0.22±0.02
Rhizobium	10	3.70±0.53	3.70±0.52	0.14±0.03	0.06±0.11	0.16±0.03	0.08±0.00
	20	3.84±0.58	4.40±0.95	0.17±0.03	0.25±0.05	0.18±0.03	0.08±0.01
	30	3.90±0.58	4.50±2.73	0.17±0.04	0.36±0.07	0.30±0.06	0.09±0.01
	40	4.10±0.37	6.20±1.96	0.19±0.05	0.51±0.10	0.42±0.08	0.10±0.01
Rhizobium with charcoal	10	3.76±0.70	4.60±0.74	0.16±0.05	0.35±0.07	0.16±0.03	0.03±0.00
	20	3.80±1.14	5.70±1.16	0.17±0.34	0.37±0.07	0.20±0.03	0.03±0.00
	30	4.10±0.37	7.76±2.44	0.18±0.01	0.40±0.07	0.19±0.03	0.83±0.16
	40	4.50±0.94	9.02±3.20	0.19±0.01	0.60±0.12	0.60±0.12	1.84±0.36
Azotobacter and *Rhizobium*	10	4.40±0.90	10.40±3.26	0.16±0.58	0.36±0.07	0.03±0.03	0.06±0.00
	20	4.90±0.24	11.00±4.64	0.19±0.33	0.47±0.09	0.03±0.05	0.07±0.01
	30	4.98±0.51	11.50±1.76	0.20±0.02	0.62±0.12	0.80±0.16	0.10±0.02
	40	5.20±0.98	12.50±1.14	0.21±0.22	0.73±0.14	0.82±0.12	0.11±0.02
Azotobacter and *Rhizobium* with charcoal	10	5.20±0.93	11.20±3.93	0.20±0.02	0.93±0.16	0.76±0.09	0.06±0.00
	20	5.70±1.23	13.00±2.52	0.20±0.07	0.93±0.19	0.77±0.10	0.08±0.00
	30	6.80±0.74	13.70±4.45	0.22±0.02	0.95±0.18	0.78±0.12	0.09±0.01
	40	7.20±0.74	14.10±2.60	0.24±0.22	1.00±0.19	0.95±0.23	0.13±0.01

revealed the presence of Gram negative cocco-bacilli in pairs and Gram negative pleomorphic rods for *Azotobacter* and *Rhizobium* respectively, confirmed by partial biochemical tests with reference to Bergey's manual (Table 2). Mass cultivation of the bacteria carried out for 5 days reported bacterial counts of 2.3×10^4 cells mL^{-1}.

The effects of different concentrations of *Azotobacter*, *Rhizobium* and their co-inocula on *in vitro* seed germination and development of *Trigonella foenum-graecum* L. on glass marble containing 1X MS medium with 4% (w/v) sucrose were investigated by coating the seeds with and without charcoal. Table 3 illustrates the results of phenotypic traits which showed the maximum shoot length, root length and fresh weight for the plantlets grown on 1X MS medium with 4% sucrose and glass marbles, when inoculated with 40% concentration of *Azotobacter*, *Rhizobium* and their co-inoculum coated

Fig. 2: (a-h) *In vitro* phenotypic trait results of seeds coated with *Azotobacter* without charcoal (IM) and with charcoal (CHA). (i-p): *In vitro* phenotypic trait results of seeds coated with *Rhizobium* without charcoal (IM) and with charcoal (CHA). Keys: 1 mL-10%, 2 mL-20%, 3 mL-30% and 4 mL-40%

with charcoal, after 15 days of incubation (Fig. 2a-p, 3a-h). The physiological estimations also supported the phenotypic traits results with maximum protein, carbohydrate and chlorophyll content (Table 3).

Field trials were conducted in green house conditions to study the influence of different concentrations of *Azotobacter* and *Rhizobium* co-inocula. Table 4 represents the results of phenotypic traits and physiological estimations which revealed that 10% of the co-inoculum supported maximum growth of the plants when the seeds were coated with charcoal (Fig 4a-*l*).

DISCUSSION

Trigonella foenum-graecum L., a medicinally important plant, grown in India, was reported to have anti-diabetic, anti-fertility, anticancer, anti-microbial and anti-parasitic and hypocholesterolaemic effects (Al-Habori and Raman, 2002). In India, fenugreek is also used as a lactation stimulant (Tiran, 2003). They are a source of saponins such as diosgenin, yamogenin, gitogenin, tigogenin and neotigogens. Other bioactive constituents of fenugreek include mucilage, volatile oils and alkaloids such as choline and trigonelline (Pribac and Ardelean, 2008).

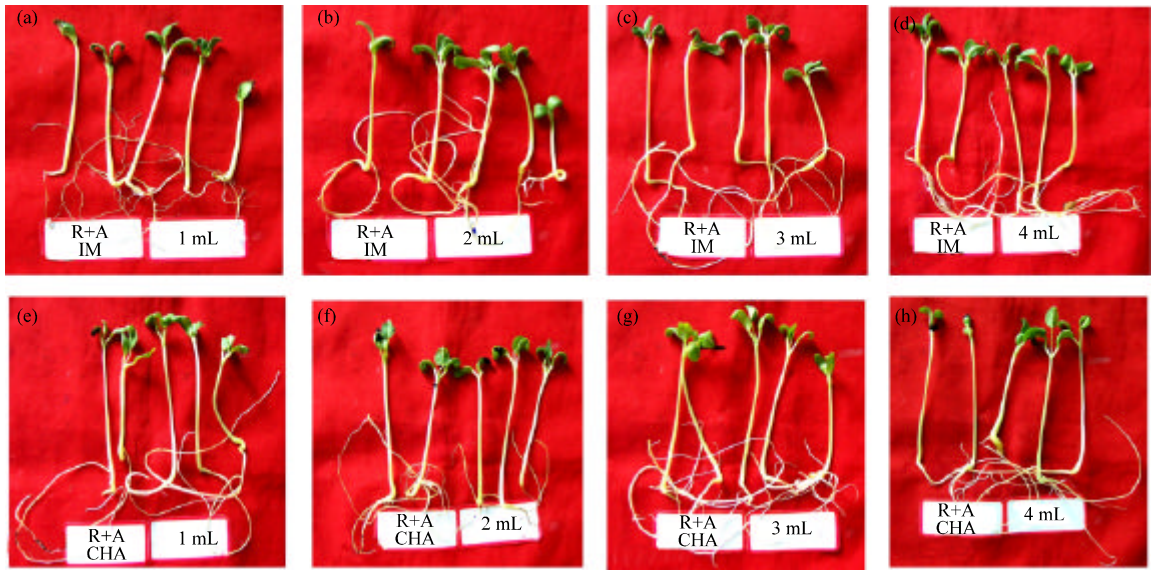

Fig. 3: (3a-h) *In vitro* phenotypic trait results of seeds coated with *Azotobacter* and *Rhizobium* co-inocula without charcoal (IM) and with charcoal (CHA). Keys: 1 mL-10%, 2 mL-20%, 3 mL-30% and 4 mL-40%

Table 4: Effects of different concentrations of *Azotobacter* and *Rhizobium* co-inocula on *in vivo* seed germination and development of *Trigonella foenum-graecum* L.

		Phenotypical traits			Physiological estimations		
Inoculum used for coating	Conc. of inoculum (%)	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Protein (mg mL^{-1})	Carbohydrate (mg mL^{-1})	Total chlorophyll (mg g^{-1})
Azotobacter and *Rhizobium*	10	8.80±1.22	21.00±2.36	0.46±0.16	0.93±0.12	0.89±0.08	0.92±0.09
	20	7.80±1.17	17.80±6.42	0.39±0.07	0.82±0.15	0.83±0.12	0.87±0.10
	30	7.60±1.46	14.40±3.30	0.34±0.05	0.66±0.13	0.82±0.12	0.72±0.00
	40	6.80±1.32	13.88±3.82	0.25±0.04	0.49±0.18	0.62±0.07	0.68±0.00
Azotobacter and *Rhizobium* with charcoal	10	9.40±1.01	24.70±2.20	0.50±0.07	0.99±0.20	0.92±0.25	0.97±0.09
	20	8.80±0.27	20.20±6.24	0.44±0.09	0.62±0.01	0.78±0.01	0.87±0.10
	30	7.40±1.18	12.00±4.00	0.40±0.05	0.53±0.00	0.76±0.03	0.83±0.00
	40	4.36±0.94	12.00±3.26	0.39±0.12	0.47±0.00	0.71±0.03	0.68±0.00

The present study was conducted to develop an *in vitro* method to study the effects of biofertilizers(*Azotobacter* and *Rhizobium*) on the seed germination and development of *Trigonella foenum graecum* L. using a simple and cost-effective liquid culture medium containing glass marbles as reusable and biologically inert support matrix alternative to conventionally used agar. These glass marbles are chemically inert, resistant to heat and action of acid and alkali. In this study, MS medium was used for regeneration of *Trigonella foenum graecum* L. based on the previous works (Pribac and Ardelean, 2008). Sucrose is the major carbon and energy source required for seed germination. Sucrose optimization studies revealed maximum growth and development of the plantlets grown on 1X MS, 4% sucrose in a liquid glass marble medium as indicated by phenotypical and physiological estimations. This result is in complete agreement with the study done by Anandarajah and McKersie (1992), who found out that embryo quality was significantly enhanced when the sucrose content of the elongation and maturation media was increased to 50 g L^{-1} from 30 g L^{-1}. The detailed results have been presented in Table 1. These results could be correlated with that of *in vitro* cultivation of *Rauwolfia serpentina* (Goel *et al*., 2009).

In the present study, *Azotobacter* and *Rhizobium* were isolated from rhizosphere soil and root nodules of *Trigonella* plants respectively, owing to the prevalence of these diazotrophs in their natural habitats. The seed inoculation with *Azotobacter* and *Rhizobium* is novel in its approach, since no previous literatures are available suggesting the use of these biofertilizers for *in vitro* studies on *Trigonella foenum-graecum* L. Mass cultivation of the bacteria carried out for 5 days reported bacterial counts of 2.3×10^4 cells mL^{-1}. The harvested bacterial cells were used to coat the seeds with and without charcoal. Charcoal was used in this study as an inert carrier to adsorb the bacterial cells effectively.

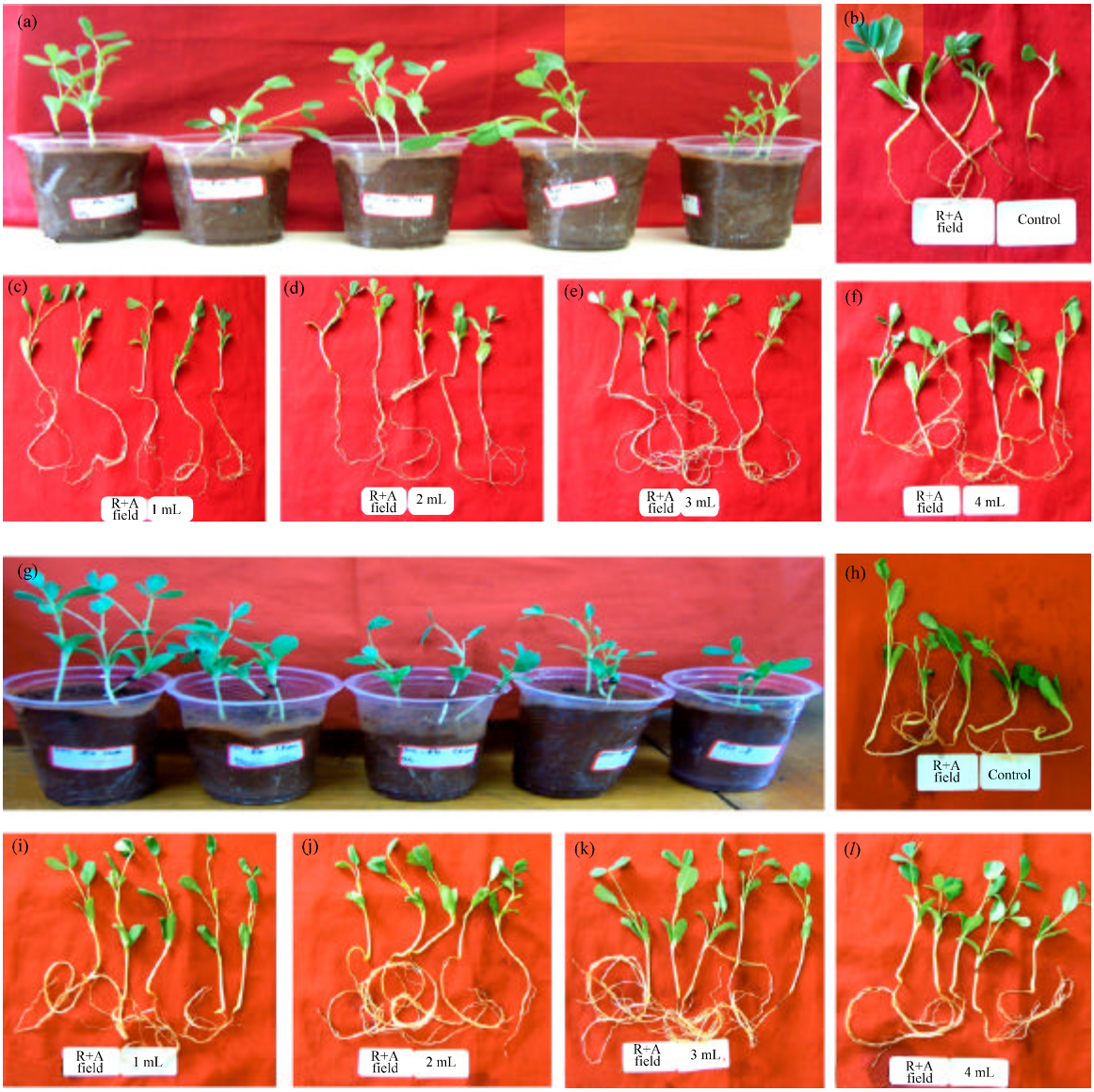

Fig. 4: (a-f) *In vivo* (field trials) phenotypic trait results of seeds coated with *Azotobacter* and *Rhizobium* co-inocula without charcoal (IM) and with charcoal (CHA). (g-*l*) *In vivo* (field trials) phenotypic trait results of seeds coated with *Azotobacter* and *Rhizobium* co-inocula without charcoal (IM) and with charcoal (CHA). Keys: 1 mL-10%, 2 mL-20%, 3 mL-30% and 4 mL-40%

Among the various concentrations of inocula used, 40% concentration of *Azotobacter*, *Rhizobium* and their co-inoculum supported maximum growth for all the seeds coated with and without charcoal. It was also interesting to note that the seeds coated with charcoal-mixed biofertilizers yielded better growth that those coated without charcoal. Similar to our study, the work done by Kim (2008), showed that the plants growing in the soil with the addition of charcoal had a higher germination rate, a greater number of leaves and grew to be taller. The physiological estimations also supported the phenotypic traits results with maximum protein, carbohydrate and chlorophyll content for the charcoal-coated seeds. The detailed results have been shown in Table 3. The development of secondary root systems was due to effective colonization of co-inoculum which might have altered the root morphology and induced the additional root hairs with the development of nodules. Another suggested mechanism which would have directly influenced the root development was phytohormones

production by the bacteria which colonized the roots of the host plants (Steenhoudt and Vanderleyden, 2000).

Field trials, conducted in this study, revealed that 10% of the co-inoculum supported maximum growth of the plants when the seeds were coated with charcoal. In comparison with the *in vitro* studies, field study has surprisingly revealed lesser concentration of co-inoculum to be more effective for plant growth. This might be due to the presence of various macronutrients and micronutrients found naturally in the soil. *Rhizobium* and *Azotobacter* are symbiotic and free living diazotrophs which had been reported to produce beneficial effects on crop yield through a variety of mechanisms including bio-synthesis of biologically active substances and modification of nutrients uptake (Somers *et al*., 2004). This can be attributed to the fact that free living diazotrophs increase the root hair density resulting in more infection sites for rhizobia, thus enhancing them by fixing the nitrogen for the legumes. Various researchers have reported the synergistic effects of auxins producing plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and yield of legume crops (Tilak *et al*., 2006). Spanik and Carlson (1996) had previously discussed the role of signal exchange between host plant and specific rhizobial species in nodule formation. In the present study, a successful attempt was made to investigate the effects of biofertilizers on *Trigonella foenum-graecum* L. under *in vitro* conditions and field study.

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