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RESEARCH ARTICLE

INFLUENCE OF DIFFERENT ADDITIVES ON SHELF LIFE OF RHIZOBIAL INOCULANTS FOR MUNGBEAN (VIGNA RADIATA L.)

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ABSTRACT

Liquid bioinoculant formulation has become the preferred technology to solve the problems associated with shorter shelf life, high contamination, poor quality, low field performance and processing solid carrier in carrier based bioinoculant formulation. In the present study, attempts were made to enhance the survival/ shelf life of liquid rhizobial inoculants (*Rhizobium* sp. strain MB703) through addition of additives such as glycerol, polyvinyl pyrollidone (PVP) and gum arabic (GA) for their ability to support growth and promote survival in yeast extract mannitol broth (YEMB) during the storage. All liquid rhizobial inoculants prepared in amended media showed higher viable count in comparison to inoculants prepared in YEMB (control) at 180 days (d) of storage. Maximum log no. of cells were obtained in inoculants prepared in YEMB amended with 2% GA followed by YEMB + 1% GA and YEMB + 2% PVP. Mungbean (*Vigna radiata* L.) seeds treated with 90 d old liquid rhizobial inoculants of strain MB703 amended with 1% PVP or 2% glycerol enhanced plant growth as compared to uninoculated control.

INTRODUCTION

Microbial inoculants represent an emerging technology designed to improve the productivity of agricultural systems in the long run. They can be seen as a technology aligned with principles of sustainable agriculture, as opposed to the increased use of pesticides and fertilizers in recent times (Kumaresan and Reetha, 2011)**.**

Biofertilizers, one of the important components of the sustainable agriculture are products containing living microorganisms which have the ability to mobilze nutritionally important elements from non- usable to usable form through biological process. These are commercially available as solid products, powder, produced from peat, as granular form, or liquid inoculants using broth medium (Albareda *et al*, 2008; Brahmaprakash and Sahu, 2012).

In carrier based inoculants, peat, wood charcoal and lignite are used as carriers and these inoculants suffer from poor quality, high contamination and unpredictable field performance. The population density of microbes in carrier based biofertilizers reduces day by day from the time of production. It is often difficult to uniformly mix peat-based inoculants with seeds. Solid-based inoculants also tend to plug precision air seeders (Singleton *et al*, 2002).

Because of these difficulties, biofertilizer/bioinoculant producers have been changing to liquid inoculant formulations instead of solid-based inoculants. Liquid biofertilizers of good quality hold great promise in agriculture because of benefits over the conventional carrier based biofertilizers such as longer shelf life, better survival on seed and better nodulation; cost saving on carrier material such as pulverization, neutralization, sterilization, contamination free and convenience of handling, storage and transportation.

Moreover, liquid inoculant coats the seeds uniformly and dries when applied through a seed auger. Seeds coated with liquid inoculant flow well when planted by using various types of seeding equipment (Tittabutr *et al*, 2007; Fernandes *et al*, 2009 and Xavier *et al*, 2010).

As a legume, mungbean is capable of utilizing atmospheric nitrogen through symbiotic association with *Bradyrhizobium* sp. (*Vigna*). Inoculation of mungbean with effective

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Bradyrhizobium inoculant is necessary for soils where the organisms are ineffective or where they are absent or scarce (Anjum *et al*, 2006; Vincent *et al*, 1962 and Bhuiyan *et al*, 2008). Most of the research work in India and abroad has been done on efficient strain selection and liquid inoculation response studies and it has been observed that it is possible to make the bacteria to survive in liquid medium for more than six months with the help of additives or cell protectants such as trehalose, arabinose, FeEDTA, glucose and polyvinyl pyrrolidone (PVP) (Tittabutr *et al*, 2007 and Daniel *et al*, 2013**).**

A break through is needed in the inoculant technology to improve the shelf life and field efficacy of biofertilizers in India to make them commercially viable and acceptable to the farmers. The present investigation was planned with the objectives to study the effect of different additives on shelf life of liquid rhizobial inoculants for mungbean (*Vigna radiata* L.) and to evaluate liquid rhizobial inoculants (amended with different additives) in mungbean crop.

MATERIALS AND METHODS

Culture, media and growth conditions

Bacterial strain such as *Rhizobium* sp. (*Vigna radiata*) strain MB703 was obtained from the Department of Microbiology, CCS Haryana Agricultural University, Hisar and seeds of mungbean crop (*Vigna radiata* L.) cv. MH 421 were obtained from the Department of Plant Breeding, CCS HAU, Hisar.

Yeast extract mannitol broth (YEMB) (Fred *et al*, 1932) was used as a basal medium for liquid rhizobial inoculant formulation with selected appropriate concentrations of additives. The fresh prepared inoculum of pure cultures of *Rhizobium* strain MB703 was used for the inoculation and transferred @ 6% to flasks containing YEMB with different additives.

Survival of mungbean Rhizobium strain MB703 in amended media

After four days of growth, cultures prepared in YEM medium with different amendments were dispensed in 100 ml sterilized plastic vials. Three vials were prepared for each treatment and all the vials were stored at room temperature.

One ml sample was drawn at 0, 30, 60, 90,120,150 and 180 days of storage, serial dilutions were prepared and plated on YEMA plates. These plates were incubated at 29 ±1 ^{0}C in a BOD incubator for 2-3 days. Colonies appearing on the plates were counted and viable cell number ml^{-1} was calculated.

Determination of shelf life of Rhizobium strain MB703

The strain was grown in YEM broth at 29±1 0C on a rotary shaker. After four days of growth, cultures of MB703 prepared in YEM were amended with either of the sterilized additives such as glycerol (1% or 2%), gum arabic (1% or 2%) and PVP (1% or 2%). The cultures containing either of the additives were transferred in sterilized plastic vials of 100 ml capacity. Rest of the procedure is same as given above.

Performance of liquid rhizobial inoculants prepared with different additives under pot house conditions

To evaluate the efficacy of liquid rhizobial inoculants, a pot experiment was conducted. Sandy loam soil was used to fill up the 5 kg earthen pots. Seeds of mungbean (*Vigna radiata* L.) cv. MH 421 were treated with different liquid inoculants stored for 90 days at room temperature. Liquid inoculant without additive was kept as control. Seeds after treatment were sown in pots. Plants were uprooted after 45 and 60 days of growth and observations on nodule number, nodule fresh weight, plant height, shoot dry weight and plant N content were recorded.

Table 1 Survival of *Rhizobium* sp. strain MB703 on supplementation of additives before the growth.

Media	Days (log no. of cfu ml^{-1})						
	0	30	60	90	120	150	180
Standard (YEMB)(M)	11.060	10.690	9.146	8.602	7.279	5.894	5.522
YEMB + 1% Glycerol (M1)	11.173	10.677	9.352	8.434	8.083	7.016	6.522
YEMB + 2% Glycerol (M2)	11.270	11.130	9.455	8.762	8.085	7.113	6.720
YEMB + 1% GA (M3)	11.055	10.732	9.568	8.779	8.166	7.020	6.513
YEMB + 2% GA(M4)	11.327	11.204	9.989	9.094	8.279	7.447	6.790
YEMB + 1% PVP (M5)	11.160	10.663	9.352	8.690	8.189	7.022	6.634
YEMB + 2% PVP (M6)	11.258	10.820	10.294	8.805	8.377	7.190	6.880
CD at 5%	0.040	0.067	0.041	0.220	0.174	0.151	0.191

M- Standard medium, M1-M6- amendments added in standard medium

Table 2 Survival of *Rhizobium* sp. strain MB703 on supplementation of additives after the growth.

Media	Days (log no. of cfu ml^{-1})						
	0	30	60	90	120	150	180
Standard (YEMB)(M)	10.563	9.135	8.954	8.145	7.262	6.954	6.823
YEMB + 1% Glycerol (M1)	10.548	9.277	9.152	8.294	7.362	7.349	7.239
YEMB + 2% Glycerol (M2)	10.567	9.364	9.269	8.303	7.447	7.404	7.271
YEMB + 1% GA (M3)	10.570	9.401	9.305	8.442	7.813	7.721	7.568
YEMB + 2% GA (M4)	10.571	9.368	9.308	8.463	7.861	7.822	7.705
YEMB + 1% PVP (M5)	10.569	9.294	9.160	8.312	7.524	7.456	7.322
YEMB + 2% PVP (M6)	10.580	9.197	9.268	8.355	7.591	7.491	7.380
CD at 5%	NS	0.082	NS	0.062	0.058	0.048	0.099

Plant biomass was determined after drying at 80 ^{0}C till constant weight. Total shoot N content was estimated by using Microkjeldhal's steam distillation method (Bremner, 1960).

RESULT

Media designated as M to M6 were used to grow the rhizobial strain. Results regarding survival exhibited that at 90 & 120 d, more than 8.0 log no. of cells were maintained in all the amended media but in YEMB (M), 8.0 log no. of cells were maintained only upto 90 d (Table 1). Maximum log no. of cells i.e. 6.880 at 180 d were observed in inoculant prepared in YEMB amended with 2% PVP (M6) followed by YEMB + 2% GA {6.790} (M4) and YEMB + 2% glycerol {6.720} (M2). Maximum log no. of cells i.e. 6.880 at 180 d were observed in inoculant prepared in YEMB amended with 2% PVP (M6) followed by YEMB + 2% GA {6.790} (M4) and YEMB + 2% glycerol {6.720} (M2). Results displayed in Table 2 revealed that in all the inoculants, viable count of *Rhizobium* sp. strain MB703 decreased with increase in storage time. At 90 d, more than 8.0 log no. of cells were maintained in all the inoculants prepared in YEMB amended with different additives and YEMB (M) (without additive). At 180 d, higher log no. of cells were observed in inoculants amended with different additives as compared to inoculant without additive (M).

Maximum log no. of cells i.e. 7.705 at 180 d were obtained in inoculant prepared in YEMB amended with 2% GA (M4) followed by YEMB + 1% GA (M3) and YEMB + 2% PVP (M6) with 7.568 and 7.380 log no. of cells ml^{-1}, respectively.

All the growth parameters were better in inoculated treatments as compared to control (uninoculated) {T1 & T2} (Fig 1). In some of the treatments inoculated with 90 d old inoculant + amendments (added before or after the growth), significantly better response was observed with respect to all growth parameters as compared to treatment inoculated with 90 d old inoculant (T4) (Table 3 & 4).

T5 - T10 = 90 d old culture of *Rhizobium* sp. strain MB703 prepared in amended YEMB, T11 – T16 = 90 d old culture of *Rhizobium* sp. strain MB703 with different amendments (added after the growth), * = nitrogen was determined only in shoot, Figures in bracket indicate % N, Recommended P added in all treatments except uninoculated control (T1). At 45 d of plant growth, among the treatments inoculated with 90 d old inoculant + amendments (added before the growth), maximum nodule weight (690 mg plant^{-1}), shoot dry weight (1.886 g plant^{-1}) and total plant nitrogen (51 mg N plant^{-1}) were observed in treatment T6 (T4 + 2% glycerol) followed by T5 (T4 + 1% glycerol); nodule weight (684 mg plant^{-1}), shoot dry weight (1.858 g plant^{-1}) and total plant N (47 mg plant^{-1}) & T8

Table 3 Effect of liquid rhizobial inoculant (MB703 strain) on mungbean crop at 45 days of plant growth

Sr. No.	Treatments	Nodule number (plant^{-1})	Nodule fresh weight (mg plant^{-1})	Plant height (cm)	Shoot dry weight (g plant^{-1})	Total plant nitrogen (mg plant^{-1})*
T1	Uninoculated control	35	309	35.0	1.216	21 (1.73)
T2	Recommended P	34	299	37.0	1.315	22 (1.70)
T3	Fresh MB703	58	508	45..0	1.641	42 (2.54)
T4	90 d old MB703	54	506	42.3	1.600	38 (2.36)
T5	90 d old MB703 + 1% Glycerol	73	684	43.0	1.858	47 (2.52)
T6	90 d old MB703 + 2% Glycerol	75	690	43.5	1.886	51 (2.71)
T7	90 d old MB703 + 1% GA	71	586	43.0	1.731	49 (2.80)
T8	90 d old MB703 + 2% GA	72	590	44.0	1.861	52 (2.80)
T9	90 d old MB703 + 1% PVP	60	521	42.6	1.670	40 (2.42)
T10	90 d old MB703 + 2% PVP	62	531	43.0	1.710	48 (2.84)
T11	90 d old MB703 + 1% Glycerol	62	540	44.0	1.763	50 (2.82)
T12	90 d old MB703 + 2% Glycerol	64	563	45.3	1.858	52 (2.82)
T13	90 d old MB703 + 1% GA	59	516	42.1	1.694	46 (2.73)
T14	90 d old MB703 + 2% GA	59	513	39.5	1.648	42 (2.56)
T15	90 d old MB703 + 1% PVP	79	695	44.6	2.027	56 (2.75)
T16	90 d old MB703 + 2% PVP	62	548	42.7	1.798	44 (2.45)
	CD at 5%	6.439	59.762	NS	0.237	

Table 4 Effect of liquid rhizobial inoculant (MB703 strain) on mungbean crop at 60 days of plant growth

Sr. No.	Treatments	Nodule number (plant^{-1})	Nodule fresh weight (mg plant^{-1})	Plant height (cm)	Shoot dry weight (g plant^{-1})	Total plant nitrogen (mg plant^{-1})*
T1	Uninoculated control	37	452	45.0	1.877	33 (1.75)
T2	Recommended P	43	486	49.0	1.959	33 (1.70)
T3	Fresh MB703	62	534	55.0	2.449	66 (2.71)
T4	90 d old MB703	56	506	51.0	2.350	56 (2.40)
T5	90 d old MB703 + 1% Glycerol	74	591	58.0	2.828	71 (2.52)
T6	90 d old MB703 + 2% Glycerol	80	640	60.3	3.307	90 (2.72)
T7	90 d old MB703 + 1% GA	73	583	56.5	2.790	78 (2.81)
T8	90 d old MB703 + 2% GA	74	591	58.3	2.828	80 (2.82)
T9	90 d old MB703 + 1% PVP	65	560	53.5	2.447	65 (2.66)
T10	90 d old MB703 + 2% PVP	64	552	53	2.409	68 (2.84)
T11	90 d old MB703 + 1% Glycerol	64	552	53.0	2.409	68 (2.82)
T12	90 d old MB703 + 2% Glycerol	65	595	55.3	2.728	77 (2.81)
T13	90 d old MB703 + 1% GA	61	526	52.7	2.409	68 (2.80)
T14	90 d old MB703 + 2% GA	62	534	55.3	2.449	69 (2.80)
T15	90 d old MB703 + 1% PVP	82	656	61.0	3.390	94 (2.78)
T16	90 d old MB703 + 2% PVP	65	595	57.7	2.727	74 (2.70)
	CD at 5%	6.564	56.693	4.643	0.271	

(T4 + 2% GA); nodule weight (590 mg plant^{-1}), shoot dry weight (1.861 g plant^{-1}) and total plant N (52 mg plant^{-1}), respectively. Among the treatments inoculated with 90 d old inoculant + amendments (added after the growth), maximum nodule weight (695 mg plant^{-1}), shoot dry weight (2.027g plant^{-1}) and total plant nitrogen (56 mg N plant^{-1}) were observed in treatment T15 (T4 + 1% PVP) followed by T12 (T4 + 2% glycerol); nodule weight (563 mgplant^{-1}), shoot dry weight (1.858 g plant^{-1}) and total plant N (52 mg plant^{-1}) & T16 (T4 + 2% PVP); nodule weight (548 mg plant$^{-1)}$, shoot dry weight (1.798 g plant^{-1}) and total plant N (44 mg N plant^{-1}), respectively (Table 3).

At 60 d of plant growth, among the treatments inoculated with 90 d old inoculant + amendments (added before the growth), maximum nodule weight (640 mg plant^{-1}), shoot dry weight (3.307 g plant^{-1}) and total plant nitrogen (90 mg N plant^{-1}) were observed in treatment T6 (T4 + 2% glycerol) followed by T8 (T4 + 2% GA); nodule weight (591 mg plant^{-1}), shoot dry weight (2.828 g plant^{-1}) and total plant N (80 mg plant^{-1}) & T5 (T4 + 1% glycerol); nodule weight (591 mg plant^{-1}), shoot dry weight (2.828 g plant^{-1}) and total plant N (71 mg plant^{-1}), respectively. Among the treatments inoculated with 90 d old inoculant + amendments (added after the growth), maximum nodule weight (656 mg plant^{-1}), shoot dry weight (3.390 g plant^{-1}) and total plant nitrogen (94 mg N plant^{-1}) were observed in treatment T15 (T4 + 1% PVP) followed by T12 (T4 + 2% glycerol); nodule weight (595 mg plant^{-1}), shoot dry weight (2.728 g plant^{-1}) and total plant N (77 mg plant^{-1}) & T16 (T4 + 2% PVP); nodule weight (595 mg plant^{-1}), shoot dry weight (2.727 g plant^{-1}) and total plant N (74 mg plant^{-1}), respectively.

In treatments T15 (T4 + 1% PVP), T12 (T4 + 2% glycerol) and T16 (T4 + 2% PVP) inoculated with 90 d old inoculant + amendments (added after the growth) significant increase in nodule weight, shoot dry weight and total plant nitrogen was observed in comparison to control T1-T4 (Table 4).

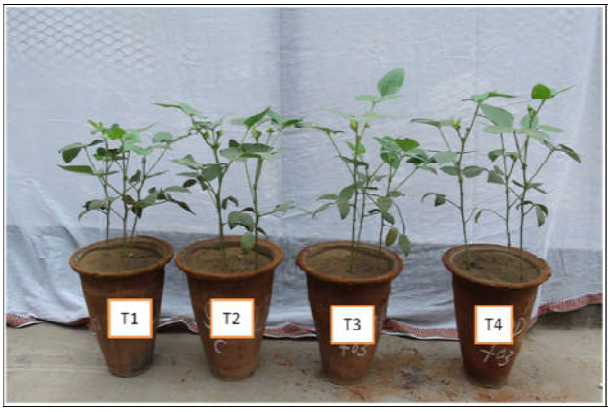

Fig 1 Effect of rhizobial inoculant (MB703 strain) on the growth of mungbean plant. T1 –Uninoculated control, T2 – Recommended P, T3 – Recommended P + Fresh MB703 inoculant, T4– Recommended P + 90 d old MB703 inoculant

DISCUSSION

Some polymeric substances, such as PVP and GA have been blended with medium for normal culturing conditions for bradyrhizobia with no adverse effect on growth. Since cells can't use these polymers as an energy source, they have other properties supporting the growth and survival of cells (Daniel *et al*, 2013). Polymers *viz*., gum arabic or PVP help in maintaining the higher viable count. Bacteria do not use these polymers as an energy sources, these polymers have other properties supporting the growth and survival of cells. PVP is believed to detoxify the fermentation media by complexing with the phenolic-type, shelf-limiting toxins in the media (Girisha *et al*, 2006). It has also protective property known as colloidal stabilization. GA has sticky consistency due to its adhesive properties which may protect the cells from desiccation and drying (Ophir and Gutnick, 1994). Glycerol may support greater number of bacteria in liquid formulation due to high water binding capacity and may protect the cells from the effect of desiccation by reducing the rate of drying (Lorda and Balatti, 1996; Temprano *et al*, 2002).

An significant increase was observed in most of growth parameters such as number of branches, number of leaves, number of nodules, root dry weight, nodule dry weight plant^{-1} of summer mungbean (*Vigna radiata* L.) due to application of biofertilizer (*Bradyrhizobium*) in a field experiment (Islam *et al*. 2006). Enhancement in plant growth parameters (shoot dry weight, nodule weight and total plant nitrogen) was more pronounced in the treatment inoculated with 90 d old inoculant amended with 1% PVP (T15) which showed maximum value of nodule weight, shoot weight and total plant nitrogen. Similar results were revealed by Prakash (2010) who evaluated liquid rhizobial inoculant technology for winter legumes and found that among different additives tested, addition of PVP or GA to liquid biofertilizer after filling in plastic bottles led to maximum nodule weight and shoot weight of inoculated plants. Many researches confirmed that inoculation of mungbean with effective rhizobial/ bradyrhizobial strains increased nodulation, dry matter production as well as seed yield (Anjum *et al*, 2006, Mansoor, 2007). Nitrogen fixing effectiveness of particular bradyrhizobial strains was the object of many investigations on mungbean crop with the aim of highly effective strain selection (Neeraj *et al*, 2008).

CONCLUSION

Survival/shelf life of *Rhizobium* sp. strain MB703 can be enhanced on supplementation of additives before/after the growth. Among all the inoculated treatments, treatment inoculated with 90 d old inoculant amended with 1% PVP (T15) showed maximum value of nodule weight, shoot weight and total plant nitrogen for the strain.

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