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Shelf Life Studies of Seed Coat Formulation of *Rhizobium* and Arbuscular Mycorrhizal Fungus (AMF) for Pulses - A New Perspective in Biofertilizer Technology

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ABSTRACT

Background: Arbuscular Mycorrhizal Fungi (AMF) and *Rhizobium* are beneficial plant partners exhibiting mutual association with crop plants. Conventional carrier based formulation has lesser population and limited shelf life. Present study was aimed to evaluate and to assess the shelf life in new formulation as well as in coated seeds under storage.

Methods: Three experiments are conducted in this study. Population of *Rhizobium* and AM fungi spore count were assessed in newer seed coat formulation under room temperature storage. Secondly survival of these organisms on the coated seeds of blackgram and greengram were evaluated. In third experiment the coated seeds stored for different duration were evaluated for germination, growth and vigour index.

Result: Rhizobial population was maintained at 10^{11} g^{-1} and AM spore load was 900-1000 spores g^{-1} up to one year of storage. AM spore count was also found sufficient up to 9 months of storage in coated seeds of both crops. Growth and vigour index were significantly enhanced in inoculated plants over uninoculated control. From the study, it was evident that these formulations can be stored upto one year at room temperature without any loss in viable count and can be used for seed coating.

Key words: AMF, Blackgram, Greengram, *Rhizobium*, Seed coat formulation.

INTRODUCTION

Pulses remain as one of the most important vegetarian diet in India. India is one of the leading countries in blackgram and greengram production. India shares 70 per cent of the total world blackgram and greengram production. *Rhizobium,* a root nodulating bacterium fixes the atmospheric nitrogen in the nodules of leguminous crops and satisfies nitrogen budget to a significant level and also contributes for the subsequent crop. The existence of arbuscular mycorrhizal fungi (AMF) in association with plant root system is known for decades and this fungal partner satisfies the crop nutritional (N, P, Fe, Zn, *etc*.) and water requirements and protects plants from biotic/a biotic stresses. In case of *Rhizobium* and AMF, plants reward their microbial partners with photosynthetically assimilated carbon (Puschel *et al*., 2017).

Biofertilizers maintain the soil health by various mechanisms *viz.*, N_2 fixation, PO_4 solubilization, K solubilization, production of plant growth regulators, *etc.,* (Sinha *et al*., 2014). Major portion of chemical fertilizers are lost into the environment when applied to soil and these biofertilizer organisms through any of the above said processes makes it available to the plants (Adesemoye and Kloepper, 2009). Hence biofertilizers remains as one of the major components of integrated nutrient management.

Advances in biofertilizer technology have led to production of liquid formulations from carrier based formulations. With an attempt to progress further in biofertilizer technology, a seed coat formulation of *Rhizobium* and spore formulation of AMF was made by the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore -3 and evaluated for its shelf life under storage and survival in coated seeds.

The most reported types of seed coating are seed dressing, film coating and pelleting. Tested in more than 50 plant species with seeds of different dimensions, forms, textures and germination types (*e.g*., cereals, vegetables, fruits, pulses and other legumes). Seed coating has been studied using various species of plant growth-promoting bacteria, rhizobia, *Trichoderma* and to a lesser extent mycorrhizal fungi. Most of the studies regarding PBM applied *via* seed coating are aimed at promoting crop growth, yield

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and crop protection against pathogens. Studies have shown that coating seeds with PBM can assist crops in improving seedling establishment and germination or achieving high yields and food quality, under reduced chemical fertilization.

MATERIALS AND METHODS

All these works were carried in the Department of Agricultural Microbiology, AC and RI, Madurai as well as in Tamil Nadu Agricultural University, Coimbatore during 2017-2019.

Survival of *Rhizobium* on seed coat formulation under storage

Powder formulation of *Rhizobium* (COC 10) was prepared with the cell load of 10^{12} cells g^{-1} and stored in polythene pouches at room temperature. *Rhizobium* was grown in yeast extract mannitol broth on large volume for 5 days till the population reaches 10^8 cfu mL^{-1}. The cultures were concentrated using TFF (tangential flow filtration) system and freeze dried. The freeze dried powder was blended with water soluble carrier dextrin and the population was standardized to 10^{12} cfu g^{-1} and the packing was done in Polythene pouches and sealed and stored under room temperature. Destructive sampling was done at monthly intervals and population was assessed up to 12 months of storage. Serial dilution and plating method with Congored Yeast Extract Mannitol Agar (YEMA) was used for assessing the survival of *Rhizobium*. Plates were incubated for 4-5 days at 28±2°C and the population was enumerated.

Survival of AM fungi on seed coat formulation under storage

AM fungi (*Rhizopagus intraradices*)was multiplied under Root organ culture in Petriplates using MSR medium and spores were harvested after dissoluting the phytagel in the medium. Harvesting was done through centrifugation. Harvested spores were blended with water soluble carrier Dextrin and the product was formulated with the spore load of 1040 g^{-1} of the product. The formulated product is packed in polyethylene pouches and stored at room temperature. Destructive sampling was done at monthly intervals and spore load was assessed up to 12 months of storage using wet sieving and decanting method as described by Gerdeman and Nicolson (1963) followed by microscopic count under stereozoom microscope.

Survival of *Rhizobium* and AM fungi in coated seeds under storage

Blackgram (var. co 6) and greengram (var. co 8) seeds were surface sterilized and treated with seed coat formulation of *Rhizobium* containing 10^{12} CFU g^{-1} and AM fungi containing spore load of 1040 g^{-1} and the seeds were packed in polythene pouches , sealed and stored at room temperature. The following are the treatments used.

Treatments

1. Single inoculation of *Rhizobium* @ 25g ha^{-1} seed (10^{12} CFU/g).
2. Single inoculation of AM fungi @ 250 g ha^{-1} seed (2.6 lakh spores).
3. Combined inoculation of *Rhizobium* and AM fungi.
4. Uninoculated control.

For each treatment 10 replications were maintained. Population of *Rhizobium* and spores of AM fungi were assessed using standard protocol at monthly intervals.

Testing the viability of coated seeds under storage

Greengram and blackgram seeds coated with *Rhizobium* and AM fungi individually as well as in combinations were evaluated for germination and vigour index.

Germination (%)

Four replicates of 100 seeds were uniformly placed on standard germination paper roll-towel medium (ROLL towel medium, ISTA, 1993) and kept in germination room maintained at 25±2°C and 90±2 per cent relative humidity. After the test period of 14 days, the seedlings were evaluated for germination percentage.

Vigour index (VI)

Vigour Index (VI) was calculated (Abdul-Baki and Anderson, 1973) by adopting the following formula after estimating shoot length and root length and expressed as whole number.

VI = Germination (%) × Mean total length of seedling in cm

On fourteenth day, ten normal seedlings per replication from roll towel medium were carefully removed at random from each treatment and the root length was measured from the base to the top of the primary root and the mean value was calculated and expressed in cm. Same way shoot length was measured from the base of the shoot to tip of primary leaf on the 14th day and the mean value was expressed in cm. For every plant total length was calculated and the mean value is derived.

RESULTS AND DISCUSSION

Survival of *Rhizobium* and AM fungi in formulation under storage

Shelf life is a very important criterion, that should be given due importance during the development of a new formulation. The success of a new product depends solely on its shelf life. The survival of *Rhizobium* and AM fungi in the newly developed formulation was assessed at monthly intervals. Initial *Rhizobium* population in the formulation was 12.6 log CFU g^{-1}. Only a slight reduction in population was observed and no significant reduction was noticed till 12 months of study and it was 11.3 log CFU g^{-1} (Table 1). The above results suggest that the newer powder formulation of *Rhizobium* can be stored up to 12 months at room temperature without any significant loss in the population. According to FCO (Fertilizer Control Order, 1985) standards the biofertilizer *Rhizobium* should contain the cell load of 5×10^7 Cells g^{-1} of a product. The product tested here had the population of *i.e.* 10^{11} cells g^{-1} and hence satisfy the FCO standards.

Many researchers have developed the formulation of *Rhizobium* with silica gel, chitosan, vermiculite, perlite *etc*., Mixture of the polymers can also be investigated as carriers (Bashan, 1998; John *et al*., 2011). Denardin and Freire (2000) reported that mixture of polymers of natural or synthetic gave the best result of maintaining the cell population for a period of 6 months. Not only the polymers are playing a role in maintaining the cell load, the form of cells what we are using for the preparation of the formulation is much important than using the fresh vegetative cells for increasing the shelf life of a product. Freeze dried cells gave the higher cell count than the recommendation so that the population loss during processing can be managed.

AM spore load in the formulation decreased from 1040 spores g^{-1} to 1000 spores g^{-1} at 6 months of storage and at the end of 12 months storage period the spore load in the formulation was 925 g^{-1} (Table 1). Though reduction was noticed it is sufficient to colonize the seedlings upon seed coating. With this count at least each seed should contain one propagule, so that it makes colonization in the roots successfully. Hence, the results suggest that the new AM formulation can be stored up to one year with the required spore load at room temperature. According to FCO, the AM inoculant should contain the minimum of 100 viable propagules g^{-1} of the product at the time of delivery. The product tested in our study recorded higher than the standard and also the population maintained during storage is also up to the standard. Formulation plays a major role on the survival of the organisms under storage and application as well as its efficiency on the crop plants (Herrmann and Lesueur, 2013). Hence it is utmost important to have better shelf life for the biofertilizers to get popularized and to have better effect on crop plants.

Efforts are made nowadays by the researchers to formulate a range of biofertilizers to ensure maximum viability (Brar *et al*., 2012). Shelf life of inoculants is a very major factor for their efficacy which mainly depends on several factors (production technology, carrier and packaging material used, transport activity) to sustain the quality of inoculants (Arora *et al*., 2010).

Our results showed the better cell viability of *Rhizobium* and AM fungi with good shelf life of one year and which are suitable for seed coating.

Survival of *Rhizobium* and AM fungi on coated seeds under storage

According to Bashan *et al*. (2014), only a few research attempts have been made on delivery mechanisms of biofertilizers. The success of a new formulation is decided by the successful delivery of the product under field conditions. With this aim, the newly developed seed coat formulation of *Rhizobium* and AMF was coated on greengram and blackgram seeds and evaluated for their survival in the coated seeds. Initial population of *Rhizobium* in greengram seed was 9.0 log CFU/g and significant reduction in population was noticed after 6 months of storage and the population at 12th month was 2.0 log CFU g^{-1} of seed. In combined inoculation treatment also initial population was 9.0 log CFU g^{-1} and here also significant reduction was noticed after 6 months of storage and the final population at 12th month was 2.5 log CFU g^{-1} of seed. With regard to AM spore load, initial count was 12.4 spores g^{-1} seed in single inoculation of AM as well as combined inoculation of *Rhizobium* and AM fungi. Though the spore count was reduced during storage it was found significant only after 6th month of storage, at 12th month the spore count was 2.2 and 2.4 g^{-1} seed respectively for single and combined inoculation.

In blackgram initial population of *Rhizobium* was 9.2 g^{-1} seed both in single as well as combined formulations and at 12th month of storage the population was 3.0 and 3.5 log CFU g^{-1} seed respectively. AM spore count was 13.2 and

Table 1: Survival of *Rhizobium* and AM fungi in powder formulation under room temperature storage.

Storage period (Month)	Population in the formulation	
	Rhizobium (COC 10)	AM Fungi
	Population per gram of formulation (log cfu/g)	spore load/g of formulation
0	12.6a	1040a
1	12.6a	1026a
2	12.6a	1032a
3	12.6a	1010a
4	12.4a	1011a
5	12.2a	1006a
6	11.9a	1000a
7	11.6a	988a
8	11.6a	970b
9	11.5a	965b
10	11.5a	950c
11	11.5a	938d
12	11.3a	925e

*Same letter in the column indicates no significant difference among the storage time.

12.8 g^{-1} seed in single and combined formulations. The spore count was reduced slowly under storage and at 12th month the spore count was 1.6 and 2.0 g^{-1} seed respectively in single and combined formulation (Table 2). The results indicated that population of *Rhizobium* as well as AMF was not significantly reduced till 6 months of storage at room temperature in single as well as combined inoculations in blackgram and greengram. Sufficient population of *Rhizobium* was maintained up to 9 months of storage in coated seeds of greengram and upto 11 months of storage in coated seeds of blackgram. AM spore count was also found sufficient upto 9 months of storage in blackgram as well as greengram.

Survival of the inoculant bacterial strains on the seed are mainly affected by desiccation, the toxic seed coat exudates as well as the higher the temperatures (Deaker *et al*., 2004). Bacterial survival on seed in turn directly affects the total yield of legume crops (Brockwell and Bottomley 1995). Muller and Berg, (2008) studied the survival of *S. plymuthica* cells on the seeds of oilseed rape applied through seed pelleting, film coating and bio-priming. They reported the survival in seed pelleting and film coating were found improved when the seeds were stored at 4°C when compared to 20°C. The survival in bioprimed seeds was unaffected irrespective of the temperature. Improper inoculant formulation or having limited survival on seed surface can influence different kinds of seed coating (O'Callaghan, 2016). The results of the present study confirmed the significant survival of the *Rhizobial* cells and AMF spores on the coated seeds of greengram and blackgram when stored at room temperature, which showed the suitability of the formulation for seed coating. Bacterial survival on seed directly affects the total legume yield (Brockwell and Bottomley 1995). However, emphases should also be given on techniques for increasing population density and survival of rhizobial strains in inoculants

Viability of coated seeds under storage

Greengram and blackgram seeds coated with *Rhizobium* and AM fungi individually as well as in combinations were evaluated for germination. In greengram, seed germination was not affected due to the coating of seed with newer formulation of *Rhizobium* and AM individually as well as in combination. It was observed as 100% in all the treatments during the initial stage of storage. No inhibition of germination was observed. Seedling vigour was significantly enhanced due to seed coating with AM and *Rhizobium* and it was found reduced significantly after 3-4 months of storage. At 12th month in control the seedling vigour was 2571 and it was enhanced to 2910 in combined inoculation of *Rhizobium* and AM fungi. Inoculation of *Rhizobium* registered an increase of 5.0% over control, AM recorded 6.4% increase over control and the combined inoculation recorded 13.2% increase in seedling vigour over uninoculated control (Table 3) at 12 months of storage. Several studies showed the positive influence of AM and *Rhizobium* on plant growth. Inoculated plant performed superior in terms of plant growth and quality over control. All plant growth and yield parameters were observed higher in plant samples inoculated with VAM and *Rhizobium* in combination as compared to single inoculation of VAM or *Rhizobium*. Dual inoculation resulted 10, 24, 17, 21 and 14% increase in seed protein. leaf chlorophyll seed fiber and ash content and number of seed pod^{-1} over single application when averaged over two years. Combined application of *Rhizobium* + VAM enhanced seed yield by 45% over control and 24% and 28% over single inoculation of VAM and *Rhizobium* respectively in lentils (Yaseen *et al*., 2016).

Table 2: Survival of *Rhizobium* and AM fungi in coated seeds of greengram and blackgram under storage

Months after seed treatment	Greengram				Blackgram			
	Population/spore load per g seed				Population/spore load per g seed			
	T_1 (R) *Rhizobium*	T_2 (A) AMF	T_3 (R+A) *Rhizobium* + AMF		T_1 (R) *Rhizobium*	T_2 (A) AMF	T_3 (R+A) *Rhizobium* + AMF	
	log cfu/g	Spore count/g	log cfu/g	Spore count/g	log cfu/g	Spore count/g	log cfu/g	Spore count/g
0	9.0a	12.4a	9.0a	12.4a	9.2a	13.2a	9.2a	12.8a
1	8.9a	11.8a	8.8a	12.4a	9.2a	12.2a	9.1a	12.6a
2	7.8a	12.0a	7.6a	12.0a	9.0a	12.0a	9.1a	12.8a
3	7.1a	11.0a	7.6a	11.6a	8.8a	10.6a	8.7a	11.6a
4	6.4a	8.4a	6.8a	8.6a	8.1a	10.0a	8.9a	10.2a
5	6.0a	8.2a	6.4a	8.0a	7.3a	8.4a	7.8a	9.0a
6	5.5a	6.8a	5.9a	7.8a	6.6a	6.2a	6.9a	6.8a
7	4.7b	5.8b	5.3b	6.6b	5.8b	5.2b	6.2b	5.6b
8	4.7b	5.6b	5.2b	6.4b	5.8b	4.2c	6.1b	4.8b
9	4.1b	5.6b	4.5c	6.2b	5.7b	4.2c	6.1b	4.2b
10	3.4c	4.4c	3.8d	5.2c	4.9c	3.0d	5.0c	3.0c
11	2.7d	3.4d	3.2e	3.8d	4.0d	1.8e	4.2d	2.0d
12	2.0e	2.2e	2.5f	2.4e	3.0e	1.6e	3.5e	2.0d

*Same letter in the column indicates no significant difference among the storage time.

In blackgram, the seed germination was not affected due to coating of seeds with *Rhizobium* and AM fungi under storage upto 12 months at room temperature. It was 100% in all the treatments at 0 day of storage. A slight reduction in seed germination was noticed in all the treatments. Generally combined inoculation registered higher germination than other treatments. No significant reduction in vigour index was observed in all the treatments up to 4 months of storage. At 12th month combined inoculation had a significant increase in vigour index over all the other treatments with an increase of 13.3 to 17.7 per cent (Table 4). The results indicated that seed germination was not affected due to the coating of seeds with *Rhizobium* and AM fungi and the vigour index was significantly enhanced in inoculated plants compared to uninoculated control. Sharon *et al*. (2015) reported that the germination of seeds is more sensitive to quality changes and is known to be affected due to storage conditions. Germination and vigour index are essential factors, which should be considered for a successful crop. Seed coating with microbial inoculants should not alter these properties of a seed. The results of the present study confirmed that the seed coating with the newer formulations of *Rhizobium* and AMF, does not affect the seed stability during the storage period. Seed coating technology have been evaluated with different crop plants such as cereals, vegetables, fruits and legumes with the use of plant growth promoting bacteria and mycorrhizal fungi and have shown that coating seeds with beneficial micro organisms can assist crops in germination, improving seedling establishment and achieving high yields and food quality, under reduced chemical fertilization (Rocha *et al*., 2019).

Seed coating with plant beneficial microorganisms is a promising technology which allows a precise application of minor amounts of inocula at the seed- soil interface (Scott, 1989) and ensuring that the inoculated organisms are readily

Table 3: Seed germination and vigour index of greengram coated with *Rhizobium* and AM fungi under storage condition.

Months after seed treatment	Germination percentage				Vigour index			
	Control	*Rhizobium*	AM	*Rhizobium* + AM	Control	*Rhizobium*	AM	*Rhizobium* + AM
0	100	100	100	100	3102	3224	3290	3491
1	96	100	97	99	3126	3205	3200	3461
2	96	100	97	99	2856	3149	3170	3480
3	96	100	98	100	2900	3126	3181	3451
4	96	97	97	98	2796	3053	3091	3320
5	96.	97	97	98	2811	2998	3015	3280
6	96	97	97	98	2776	2910	2940	3110
7	95	95	97	98	2693	2861	2980	3105
8	93	95	97	98	2653	2815	2881	3010
9	93	95	97	97	2580	2790	2835	2971
10	93	95	96	97	2623	2767	2832	2930
11	93	95	96	97	2564	2740	2780	2910
12	93	94	96	97	2571	2700	2735	2910
Cd (0.05)	NS	NS	NS	NS	94	101	117	219

Table 4: Seed germination and vigour index of blackgram coated with *Rhizobium* and AM fungi under storage condition.

Months after seed treatment	Germination percentage				Vigour index			
	Control	*Rhizobium*	AM	*Rhizobium* + AM	Control	*Rhizobium*	AM	*Rhizobium* + AM
0	100	100	100	100	2891	3009	3072	3453
1	98	99	99	100	2875	2989	3045	3440
2	98	98	98	99	2894	2978	3009	3399
3	98	98	99	99	2910	2960	3024	3369
4	96	98	98	98	2858	2963	3021	3350
5	96	97	98	98	2878	2939	3003	3335
6	93	96	96	97	2753	2913	2947	3291
7	92	93	93	94	2759	2821	2831	3286
8	91	92	91	92	2717	2788	2801	3275
9	88	90	89	90	2690	2751	2745	3270
10	87	88	88	89	2670	2669	2700	3137
11	86	88	89	90	2645	2660	2720	3100
12	86	90	87	88	2600	2660	2700	3060
Cd (0.05)	NS	NS	NS	NS	120	47	88	77

accessible at germination as well as early developmental stages of the plant, stimulating healthy and rapid establishment and consequently maximizing the crop production (Colla *et al*., 2015). Further seed coating significantly reduce the amount of inoculum needed as well as avoiding the wastage of inoculum through non targetted delivery. Also left over after seed treatment can be reduced through this new formulation since it coats the seed very thin.

CONCLUSION

Shelf life and stability are the two perspectives that should be considered during the development of a new biofertilizer formulation. From the present study it was evident that the newer freeze dried powder formulation of *Rhizobium* and AMF can be stored up to 12 months at room temperature without any significant loss in the population and spore load respectively. The outcome of the current research concludes that, this new seed coat formulation of *Rhizobium* and AMF may serve as forward milestone in the biofertilizer technology. In future many more newer formulations which ensure the reachability of the seed and the rhizosphere, as well as the fortification of the formulation with stimulating compounds such as flavanoids, precursors and plant hormones are need to be worked out.

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