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Shelf life of bioagents and longevity of biologically coated pigeonpea seed

V Jagadeesh[1], Sujatha Patta[1]*, S Triveni[2], K Keshavulu[1], K Jhansi Rani[1] & K Raghavendra[3]

[1] Department of Seed Science and Technology; [2]Department of Agriculture Microbiology and Bio-energy, Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad-500 030, India
[3]Centor India, Hyderabad-500 084, Telangana

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Biological seed coating is a new technique of seed treatment through which biological agents are coated over the seed surface for effective control of seed and soil-borne pathogens. In this study, pigeonpea seed was biologically coated with *Pseudomonas fluorescens*, *Rhizobium* spp. and Phosphorus solubilizing bacteria (PSB) using biofriendly polymer and sugar syrup as adjuvants. The shelf life of bioagents and seed quality parameters was studied during six months of storage period. The colony units of *Pseudomonas* increased with biofriendly polymer either as individual or in consortia with biofertilizers. Six months after treatment, more colony units of *Pseudomonas fluorescens* were recorded on the surface of biologically coated seed of pigeonpea with biofriendly polymer as an adjuvant compared to sugar syrup. Seeds coated with *Pseudomonas* and PSB using biofriendly polymer recorded high seed germination and seedling vigour compared to sugar syrup. The observations reveal that there is a possibility of coating seed with biological agents using biofriendly polymer immediately after processing or before packaging without affecting the shelf life of bioagents and seed quality. Thus, the biologically coated pigeonpea seed in advance of cropping season can go a long way in minimizing risk associated with on farm seed treatment.

Keywords: Bioagents, Biopolymer, *Cajanus cajan*, Phosporus solubilizing bacteria, Seed coating

Pigeonpea [*Cajanus cajan* (L.)] is an important pulse crop rich in protein content (22%) which is almost three times that of cereals. Keeping the importance of pulses in view, the 68th United Nations General Assembly of United Nations Organization (UNO), Geneva declared 2016 as the International Year of Pulses (IYP)[1] with the motto of "Nutritious seeds for a sustainable future". The major constraints in obtaining potential yield of pigeonpea are the incidence of diseases and insects. Among many other pathogens, pigeonpea wilt caused by *Fusarium udum* is the major disease in India. The disease may appear during early stages of plant growth (4-6 wk old plant) and drastically influences the crop yield by poor field emergence, seedling establishment and plant stand. The incidence of wilt results in yield loss up to the tune of 18.86-54.24% in pigeonpea[2]. Public concerns with fungicide residues, as well as pathogen resistance to some pesticides, have increased the need to find alternative methods like biological control for protection against crop diseases[3,4].

*Correspondence:
Phone: +91 9491128885 (Mob.)
E-mail: patta.sujatha@gmail.com

Demand for biological seed treatment is growing rapidly with a speculation of capturing as much as 20% of the global seed treatment market[5]. Biological seed coating refers to the application of certain biological agents to the seed prior to sowing in order to suppress, control or repel pathogens, insects and other pests that attack seeds, seedlings or plants and its ranges from a basic dressing to coating and pelleting[6]. Application of beneficial microorganisms to seeds is an efficient mechanism for placement of microbial inoculum into soil where they will be well positioned to colonize seedling roots and protect against soil-borne diseases[7,8] and it also gives protection against oxidative stress induced by heavy metals[9,10]. Soil application of *Trichoderma viride* + seed treatment shows minimum wilt incidence with maximum yield[11]. The polymer acts as a protective cover for bioagents, helps in improving the shelf life and dust free seed[12]. The biological seed treatment is low cost, alternative viable technology[13] to chemical based plant protection and nutrition. Conventionally the seed treatment with bioagents is done a day before sowing as mostly on farms on the pretext that the bioagent will lose the viability during seed storage if the treatment is done during seed processing and packaging. Often the farmers fail to treat the seed

with bioagents due to lack of knowledge and also proper adjuvant. As sufficient literature is not available regarding shelf life of bioagents on the surface of coated seed, here, we studied the effect of bioagents on seed longevity during storage and an effective adjuvant for biological seed coating in pigeonpea in order to standardize the technology.

Material and Methods

The present investigation was carried out during *Kharif*, 2015 and *Rabi*, 2016 in the Department of Seed Science and Technology and Department of Agricultural Microbiology and Bio-energy, College of Agriculture, Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad, Telangana. Freshly harvested seeds of Pigeonpea variety LRG-41 were collected from Regional Agricultural Research Station, Lam, Guntur, Andhra Pradesh. The seed possessed initial germination of 85%, electrical conductivity of seed leachates as 12.12 µmhos/cm and seed moisture content of 8.1%. Biological agents like *Pseudomonas fluorescens*, *Rhizobium* spp. and Phosphorous solubilizing bacteria (PSB) were obtained from Agri Biotech Foundation (ABF), Rajendranagar, Hyderabad and Telangana. Biofriendly polymer was collected from Centor India, Secunderabad, Telangana.

Pigeonpea seed was coated with bioagents at the rate of 12 g kg^{-1} seed using adjuvants (either biofriendly polymer or sugar syrup) @ 6 g kg^{-1}. For combined inoculation, two biological agents were used at the rate of 6 g each. Uniformly coated seed was shade dried for 2 h. Treated seed was made into 3 replications and packed in sealed polythene bags and stored under ambient conditions.

Treatments

Selected seeds were made into nine groups (T1-T9) and groups T1 to T8 were given the following treatments [T1: Seed + *Pseudomonas fluorescens*+ Biofriendly polymer (BF polymer); T2: seed + *Pseudomonas fluorescens*+ sugar syrup; T3: seed + *P. fluorescens*+*Rhizobium* + BF polymer; T4: seed + *P. fluorescens*+*Rhizobium* + sugar syrup; T5:seed + *P. fluorescens*+ Phosphorus solubilizing bacteria (PSB) + BF polymer; T6: seed + *P. fluorescens*+ PSB + sugar syrup; T7*: seed + *P. fluorescens*+ Biofriendly polymer; T8: seed + Thiram], respectively. Group T9 with seeds only were kept as untreated control. [*Farmer practice of seed inoculation as one day before sowing (fresh treatment every month)].

The viability and shelf life of biological agents was evaluated using standard plate count agar method. One gram of treated seed samples were serially diluted and appropriate dilutions were plated on King's B medium (*P. fluorescens* treated samples), Yeast extract mannitol agar medium (*Rhizobium* treated samples) or Pikovskaya medium (PSB treated samples). Three replications were maintained for each dilution plated. The plates were incubated for 2 days at 28℃, following which the counts were taken.

The standard germination test was conducted as per International Seed Testing Association (ISTA) rules[14] by adopting between paper method. The seedling length was measured from 10 randomly selected seedlings on 6th day of germination test. Dry weight of 10 normal randomly selected seedlings from each replication was estimated by oven drying at 80±1℃ temperature for 24 h as per ISTA rules[14]. The data recorded were analyzed statistically by adopting completely randomized design (CRD)[15] and the standard error of difference was calculated at 5% probability level to compare the mean difference among the treatments. The data recorded as percentage were transformed to the respective angular (arc sin) values before subjecting them to statistical analysis.

Results

Effect of biofriendly polymer on viability of *Pseudomonas fluorescence, Rhizobium* and Phosphorus solubilizing bacteria (PSB)

Significant variability was observed with biofriendly polymer for colony forming units of *Pseudomonas fluorescens*, *Rhizobium* and Phosphorus solubilizing bacteria (Table. 1). *P. fluorescens* and PSB recorded comparatively more number of colony counts with biofriendly polymer (12.6 and 13.8×10^5 cfu g^{-1}, respectively) than their commercial formulation (11.9 and 12.2×10^5 cfu g^{-1}, respectively). *P. fluorescens* showed higher number of colony units in its consortia with *Rhizobium* spp. or PSB (18.7 or 19.0×10^5 cfu g^{-1}, respectively) compared to its commercial formulation (12.6×10^5 cfu g^{-1}). But in these consortia, both *Rhizobium* spp. and PSB showed a reduction in colony counts (12.4 and 12.1×10^5 cfu g^{-1}, respectively) compared to their individual commercial formulations (16.3 and 13.8×10^5 cfu g^{-1}, respectively). This might be due to compatibility and antagonistic reactions of *P. fluorescens* and biofertilizer.

Effect of adjuvants and type of inoculation on shelf life of *P. fluorescens* on biologically coated pigeonpea seed

There was a significant difference among the adjuvants on the colony forming units (CFU) of *Pseudomonas fluorescens* throughout the storage period of six months (Table 1). Over a period of six months, the initial mean CFU count of 12.4×10^5 cfu g^{-1} seed was reduced to 0.9×10^5 cfu g^{-1} seed. *Pseudomonas* recorded doubled colony units (16.3×10^5 cfu g^{-1} seed) with biofriendly polymer compared to sugar syrup (5.9×10^5 cfu g^{-1} seed). Six months after treatment, *Pseudomonas* with biofriendly polymer recorded very high colony count (1.6×10^5 cfu g^{-1} seed). Biofriendly polymer showed higher average CFU (15.3×10^5 cfu g^{-1}seed) compared to farmer practice of sugar syrup (8.6×10^5 cfu g^{-1} seed) and similar trend was recorded throughout the storage period. Irrespective of adjuvants, a drastic drop in the CFU count was observed after second month of storage. But at the end of six months of storage period, high number of colony counts were observed with bio friendly polymer (1.1×10^5cfu g^{-1}seed) compared to sugar syrup (0.8×10^5cfu g^{-1}seed). With regard to the effect of type of inoculation, *P. fluorescens* recorded more colony units in its single inoculation (16.3×10^5cfu g^{-1}) compared to its count in the consortia either with *Rhizobium* or PSB (14.4×10^5 cfu g^{-1}) and similar trend was observed throughout the storage period. Irrespective of type of inoculation, a drastic drop in the colony count was observed from the second month after storage. However, after six months of storage, high mean number of colonies were observed (1.6×10^5 cfu g^{-1}seed) with single inoculation. This might be due to the reduction in the dosage of bioagents used for combined inoculations.

Effect of biological seed coating with *Pseudomonas, Rhizobium* and PSB on seed quality and longevity of pigeonpea

Biological seed coating showed significant effect on seed germination, seedling length and longevity of pigeonpea (Table 2 and Fig. 1). Irrespective of treatments, there was a decrease in the mean seed germination, seedling length and seedling dry weight over a period of six months of storage (10%, 10.8 cm and 0.077 g, respectively). Pigeonpea seeds coated with *Pseudomonas*, PSB and biofriendly polymer (T5) maintained significantly higher germination, seedling length and seedling dry weight (77%, 15.3 cm and 0.897 g, respectively) compared to other treatments. Among seed coating materials, irrespective of bioagent, seed germination recorded with bio-friendly polymer was on par with that of chemical protectant and untreated control (75%) (Table 2). The sugar syrup as an adjuvant recorded low seed germination, seedling length and seedling dry weight (65%, 13 cm and 0.87 g, respectively) which is lower than the prescribed Indian Minimum Seed Certification Standards (75%) for pigeonpea (Table 2).

Biological seed coating with single biological organism of *P. fluorescens*, showed slightly higher seed germination compared to combined inoculation of *P. fluorescens* with *Rhizobium* or PSB but this effect was nullified after six months of storage. Consortia of *P. fluorescens* with *Rhizobium/PSB* showed more seedling length (14.90 cm) and seedling dry weight (0.891 g) compared to seed coating with only *P. fluorescens* (13 cm and 0.860 g, respectively) (Table 2).

Table 1 — Effect of adjuvants on viability and shelf life (CFU) of *Pseudomonas fluorescens* during storage of biologically coated seed of pigeonpea

Treatment	*Pseudomonas fluorescens* x 10^5 cfu g^{-1} seed						
	0MAS	1MAS	2MAS	3MAS	4MAS	5MAS	6MAS
T1	16.3	15.8	5.2	4.7	3.4	2.2	1.6
T2	5.9	4.8	4.7	2.3	1.7	1.7	1.4
T3	12.4	7.1	5.4	2.9	2.4	1.4	1.0
T4	11.6	5.0	4.8	2.0	1.8	1.0	0.7
T5	16.3	7.5	5.5	1.3	0.9	0.6	0.3
T6	8.3	6.1	4.2	1.2	0.9	0.4	0.2
T7	16.3	15.7	7.6	2.6	2.6	1.9	1.7
Mean	12.4	8.9	5.3	2.4	1.96	1.3	0.9
S.Em	0.271	0.265	0.180	0.125	0.137	0.135	0.130
S.Ed	0.384	0.375	0.255	0.177	0.194	0.191	0.183
CD (0.05)	0.831	0.812	0.552	0.384	0.419	0.414	0.397
C.V	4.273	6.191	5.848	8.940	12.112	17.805	22.793

[T1, seed+*Pseudomonas fluorescens*+biopolymer; T2, seed+*P. fluorescens*+sugar syrup; T3, seed + *P. fluorescens* +*Rhizobium* + biopolymer; T4, seed + *P. fluorescens* +*Rhizobium* + sugar syrup; T5, seed + *P. fluorescens* +PSB + biopolymer; T6, seed + *P. fluorescens* +PSB + sugar syrup; T7, seed + *P. fluorescens* +biopolymer (fresh treatment); and MAS, Months After Storage]

Table 2 — Effect of biological seed coating with *Pseudomonas fluorescens*, *Rhizobium* and Phosphorus solubilizing bacteria on seed germination (%), seedling length (cm), seedling dry weight (g) and storability of pigeonpea

	Seed germination (%),				Seedling length (cm)				Seedling dry weight (g)			
Treatment	0 MAS	2 MAS	4 MAS	6 MAS	0 MAS	2 MAS	4 MAS	6 MAS	0 MAS	2 MAS	4 MAS	6 MAS
T_1	81 (63.92)	80(63.19)	77(61.11)	75 (56.99)	23.2	21.2	17.5	13.7	1.024	1.011	0.985	0.849
T_2	83 (65.41)	81(64.42)	78(62.27)	68 (56.58)	26.2	24.4	17.4	14.2	0.972	0.963	0.935	0.888
T_3	79 (62.73)	78(62.03)	75(60.00)	72(54.75)	25.4	22.7	16.2	14.5	0.953	0.951	0.928	0.884
T_4	79 (62.73)	78(62.03)	75(60.00)	65(53.55)	25.7	20.1	14.4	13.1	0.971	0.930	0.884	0.865
T_5	83 (65.40)	82(64.65)	78(62.26)	77(61.35)	25.5	22.7	17.8	15.3	0.956	0.951	0.934	0.897
T_6	78 (62.04)	77(61.35)	73(58.91)	62 (54.42)	22.9	21.3	18.3	13.0	0.991	0.970	0.964	0.893
T_7	84 (66.17)	81(64.16)	78(62.03)	74 (57.21)	25.3	20.7	13.1	13.3	1.030	0.916	0.868	0.872
T_8	84 (66.43)	81(64.45)	81(63.92)	75 (62.50)	25.1	20.8	17.1	14.8	0.950	0.950	0.930	0.873
T_9	84 (66.18)	81(63.94)	80(63.44)	75 (61.57)	23.9	21.3	15.5	15.0	0.946	0.943	0.877	0.870
Mean	81 (63.92)	80(63.20)	77(61.11)	71 (60.88)	24.8	21.7	16.4	14.0	0.977	0.954	0.923	0.877
S.Em	0.502	0.702	0.488	1.140	0.575	0.716	0.563	0.235	0.023	0.029	0.026	0.026
S.Ed	0.709	0.992	0.691	1.613	0.813	1.013	0.796	0.333	0.033	0.041	0.037	0.037
CD (0.05)	1.490	2.085	1.452	3.414	1.721	2.144	1.685	0.704	N.S	N.S	N.S	N.S
C.V	1.346	1.919	1.376	3.423	4.013	5.717	5.961	2.905	4.055	5.202	4.865	4.865

[MAS, Months After Storage]

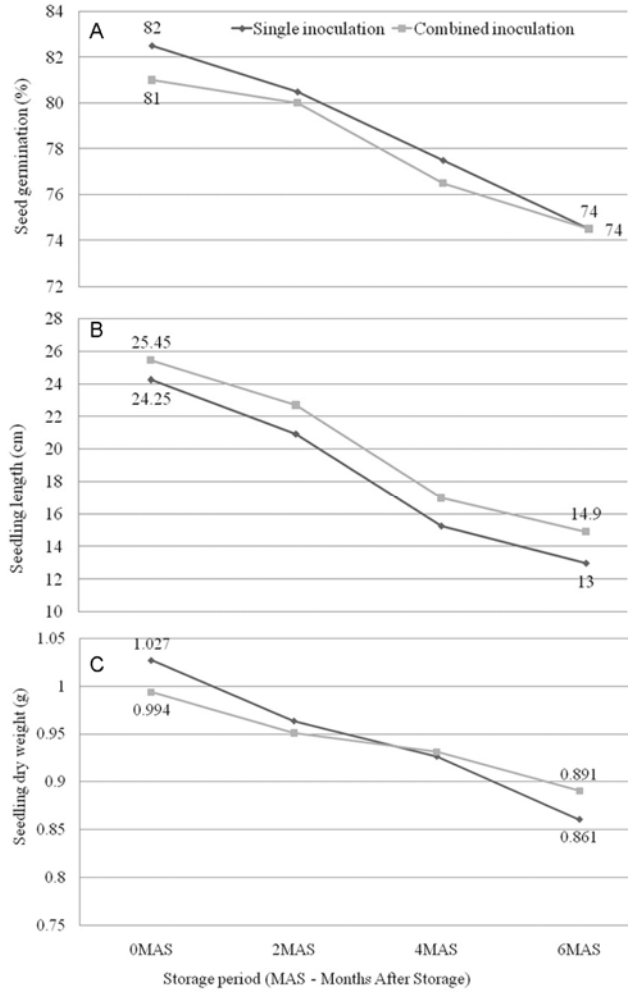

Fig. 1 — Effect of type of inoculation along with *Pseudomonas* and biofertilizerson seed germination on different parameters in biologically coated pigeonpea seed. (A) seed germination (%); (B) seedling length (cm); and (C) seedling dry weight (g)

Discussion

Biological control of plant diseases is safe alternative technique to chemical methods[5]. This is gaining attention due to increased public concern about the use of fungicides for crop protection and development of pathogen resistance[16]. Beneficial microbes, including antagonistic bacteria and fungi, applied as seed treatments provide unique opportunities and benefits for crop protection, especially for protection against soil borne fungal pathogens by growing and proliferating on germinating seeds, and may eventually colonize the whole root system or rhizosphere[17]. Survival and multiplication of microorganisms depends upon the carrier material used either for commercial formulation or for coating/inoculation[18]. So far, it was a practice of on-farm application of biological organisms on to the seed surface prior to sowing owing to weak binding nature of adjuvants like starch, sugar or jaggery syrup leading to dust off problem. Moreover, this on-farm practice demands time and attention from the farmer during peak sowing time, because of which this technology might not be followed by many farmers unable to tap the potentiality of bioagents during seedling emergence. We made an attempt to address this problem so that biological seed coating technology can be introduced in the seed supply chain itself. We found that *P. fluorescens* and PSB showed higher number of colony units with biofriendly polymer than its commercial formulation but a reverse trend was observed in *Rhizobium*. This might be due to the fact that the composition of biopolymer might have

enhanced the rate of cell multiplication in *Pseudomonas* and PSB. The cell multiplication rate of microorganism may be influenced by the carrier material[18]. The consortia of *Pseudomonas* with *Rhizobium* or PSB showed an increased colony count of *Pseudomonas* but the same consortia reported reduction in the colony counts of both the biofertilizers in their commercial formulations. This might be due to the fact that *Pseudomonas* might have some suppressive reaction on the cell multiplication rate of either on *Rhizobium* or PSB. This might also be due to compatibility and antagonistic reactions of bio control agent and bio fertilizer. This research gap needs to be addressed in detail.

Biofriendly polymer showed higher average colony units compared to farmer practice sugar syrup and similar trend was recorded throughout the storage period of six months[19]. Chemical composition of biofriendly polymer might have some growth promoters in addition to sucrose; this might have contributed for the rapid multiplication of organism in addition to its adhesive nature. This is a specialized polymer designed to give greater protection when applied with rhizobia, either in a peat or liquid form counts remain high in slurry mixtures for over 4 h when polymer and peat is mixed[20]. Similarly, O'callaghan *et al.*[8] reported that *P. fluorescens* strain F113 application to onion seed using patented biopolymer technology maintained high cell numbers on seed stored at 4℃ for up to 70 days. Bacterial numbers declined on seed stored at 20℃, but significant numbers of bacteria remained viable after 70 days storage. They also reported that the packaging material had a significant effect on bacterial survival on seed. Their study has demonstrated the potential to treat seed with fluorescent *pseudomonads* with biocontrol capability. With regard to the effect of type of inoculation, biological seed inoculation with bio friendly polymer and *Pseudomonas fluorescens* in single inoculation recorded high colony count compared to its consortia with *Rhizobium* or PSB. This might be due to the restricted multiplication rate of *Pseudomonas* due to competition for food and space by other organisms in the consortia[13,21]. The reduced dosage of organism used for treatment in consortia to half compared to the treatment with individual organism might have contributed for these results.

Six months after storage, biological seed coating of *Pseudomonas*, PSB with biofriendly polymer maintained significantly higher germination percentage compared to the same consortia with sugar syrup[22,23]. This was in conformity with the findings in soybean that polymer-coated seeds in general deteriorated at a slower rate compared with the control and recorded high germination[24]. In another finding, Harish *et al.*[25] also reported in *Cajanus cajan* (L.) that the combinations of *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 with half dose of chemical fertilizers showed a significant increase in seed germination (94%). Six months after treatment, biological seed coating of *Pseudomonas* and PSB with bio friendly polymer recorded more seedling length and seedling dry weight followed by untreated control. The consortia of *Pseudomonas* and PSB along with bio-friendly polymer might have enhanced the nutrient availability to the developing seedling during cell division and multiplication resulted in increased seedling length (cm) and seedling dry weight (g). With regard to the effect of coating material on seed longevity, bio friendly polymer as an adjuvant showed on par germination with seeds coated with fungicide and untreated control after six months of treatment but sugar syrup recorded low seed germination. This indicates that there was no reduction in seed germination with bio friendly polymer. Similar findings were reported by Giang and Gowda[26] in paddy, Shakuntala *et al.*[27] in sunflower, Wilson and Geneve[28] in corn and Zhang Li[29] in wheat and corn seeds as an increase in seed germination in the polymer coated seeds compared to untreated control by reducing the number of abnormal seedlings.

Consortia of *Pseudomonas fluorescens* and *Rhizobium/PSB* showed more seedling length and seedling dry weight compared to seed coating with *Pseudomonas* alone. Similarly, Sanjukta and Anusuya[30] reported in fenugreek that combined inoculation with phosphor compost and bioinoculants like *Frateuria aurentia* (potassium mobilizer), *Trichoderma viride*, *Rhizobium* spp. and *P. fluorescens* significantly increased plant biomass, yield and nutrient content. Nazir *et al.*[31] investigated in black gram (*Vigna mungo* L.) that the combined inoculation of *Rhizobium* + PSB slightly improved growth characters. Tagore *et al.*[32] reported in chickpea that the better effect of *Rhizobium* and Phosphate solubilizing bacterial inoculants on shoot dry weight, yield attributes and yield.

Thus, the biologically coated pigeonpea seed with biopolymer retained seed quality up to six months of

storage owing to survival of bioagents. Consortia of bioagents also better survived during storage. These findings may pave a way to further studies with different bioagents and crops to study the shelf life and the efficacy in storage and agronomic performance of crop after sowing.

Conclusion

These findings may be helpful in the standardization of dry inoculated technology as a best alternative to on-farm treatments. Seed can be treated with biologicals well in advance of sowing i.e., after seed processing or before packaging. Biological seed coating with consortia of bioagents may be advisable for best results and long duration of protection.

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Conflict of interest

The authors declare no conflict of interest.

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