Original Research Article

Assessment of shelf-life and efficacy of the seed-coating delivery system of biofertilizers in maize

ABSTRACT

Film-coating of seeds is a widely accepted technology in agriculture for effectively protecting germinating seeds and seedlings against biotic and abiotic stresses and successfully delivering agro-chemicals, biomolecules, and bio-inoculants to the rhizosphere region. The present experiments explored the possibility of using hydroxypropyl methylcellulose (HPMC) and dextrin as film-coating agents for impregnating maize seed with four different biofertilizer strains. *Azospirillumbrasilense* (Sp7), *Bacillus megaterium*var*phosphaticum* (Pb1), *Paenibacillusmucilaginosus*(KRB9), and *Pseudomonas chlororaphis* (ZSB15) were prepared as consortium containing an equimolar ratio of each strain. The *in vitro* growth experiment revealed that HPMC, dextrin, or a combination of both did not affect the growth of all four biofertilizers. When biofertilizer consortium was film-coated on maize seed using HPMC and dextrin as film-coating /binding and protecting agents, no apparent changes were observed in the size, shape, weight, and volume of maize seeds. The shelf-life study on film-coated maize seeds revealed that the microbial inoculants accounted for a one-log reduction (from 10^4 cells at initial to 10^3 cells after 3 months) when stored at low temperature. The pre-coated seeds with microbial-inoculants showed significant improvement in seedling growth. Hence, the film-coating of maize seed with microbial inoculants using HPMC and dextrin could be an effective delivery system for ensuring rhizosphere colonization of biofertilizers for sustainable maize cultivation.

Keywords:*Biofertilizers; Film-coating; Polymers; Seed coating; Maize; Shelf life*

1. INTRODUCTION

Biofertilizers are the natural, economical, environmentally safe, and effective alternative bio input against the high-cost synthetic fertilizers. Continuous use of biofertilizers also aids in the conservation of soil structure and biodiversity of agricultural land [1]. Biofertilizers are typically formulated with one or more beneficial microorganisms that facilitate efficient nutrient uptake by biological processes such as nitrogen fixation and solubilization of insoluble nutrients. In addition, they promote crop growth by secretion of plant growth promoting substances, production of antibiotics, and improve the soil structure by decomposing organic matter in the soil. Therefore, biofertilizers act as an ideal input for organic farming and conservation farming and are accessible to marginal and small farmers over chemical fertilizers [2]. Due to increasing emphasis on the use of organic inputs and the increasing support from the government to boost biofertilizer production across the world, it

is estimated that the global biofertilizer market will be USD 4.47 billion in 2029 from USD 2.02 billion (2022) with an annual growth rate of around 12% [3].

Even though biofertilizers are eco-friendly and cost-effective, the widespread use of biofertilizers among farmers is restricted by many constraints arising from the lab to field level. The significant constraints of biofertilizers are the shelf-life and their effective delivery to the crop's niche [4, 5]. The efficiency of biofertilizers is drastically reduced due to prolonged storage [6]. Soil application is generally used for delivering a large quantity of microbial inoculants which protect the seeds from inhibitory compounds produced by the seeds (e.g., antimicrobial compounds). This method is usually done at the time of sowing by using a lid, liquid, or encapsulated formulated inoculants [7, 8]. Yet, this method requires a large quantity of inoculants, making them non-economical. The most common method normally followed by farmers is seed treatment using adjuvants like rice gruel just before sowing. However, the on-farm practice of seed treatment might lead to a dust-off problem, resulting in insufficient cell load for effective colonization of plant roots [9, 10]. Maintaining a high cell load on the seed surface is a great challenge since a high cell load of bio-inoculants will ensure the competency of biofertilizer strain with the native soil microflora for root colonization. At this juncture, the application of biofertilizers through seed coating is the appropriate approach that delivers the bio-inoculants cells directly to the targeted plants' rhizosphere region, also making this a cost-effective method for large-scale field application of inoculants [6, 11].

Coating the seeds with beneficial microorganisms allows the precise application of a small quantity of inoculants at the seed–soil interface [12]. This approach ensures that the microbes are readily accessible during seed germination and early plant development. The effective colonization of inoculated strains will promote healthy and rapid crop establishment, thereby boosting the growth and yield [13]. The microbial seed coating approach has been employed in vegetable crops (onion, beetroot, carrot, tomato), cereals (sorghum, wheat, maize), oilseed crops (rapeseed, sesame) and legumes (soybean, alfalfa, cowpea). These studies reported that seed coating of plant growth promoting rhizobacteria improved their survival rate on seeds and seed germination, nutrient acquisition, nodulation, plant growth and yield. The components of biofertilizer-seed coating include the inoculants strain, binding agents or polymer, and a colorant. The polymer used should be thin, easy to apply, hydrophobic toward water vapor but readily diffusing in soil upon contact with water to enable seed germination. In particular, it should not be toxic to biofertilizerstrain/seed/seedlings during germination [14]. Coating bioinoculants over the seed surface using a binding agent improves the adherence of bioinoculant to the seed, assuring dust-free handling [15].

Besides, the seed coating technology facilitates a precise distribution of active substances on the seed surface without affecting the form of the seed and with a weight gain of not more than 2 per cent[16]. Furthermore, polymer-coated seeds can be stored for an extended period if the seeds are maintained under appropriate storage conditions [17]. Hence, delivering the biofertilizers through seed coating would be a potential tool for sustainable agriculture, as the technology can precisely deliver the recommended biofertilizer strain in the rhizosphere with a low amount of inocula[18]. In the present work, we attempted to deliver four different beneficial microorganisms through seed coating in maize and assessed their shelf-life and bio-efficacy.

2. MATERIAL AND METHODS

2.1 BIOFERTILIZER STRAINS USED FOR SEED COATING OF MAIZE

Four bacterial biofertilizer strains viz., *Azospirillumbrasilense*(Sp7), *Bacillus megateriumvar. phosphaticum* (Pb1), *Paenibacillusmucilaginosus* (KRB9), and *Pseudomonas chlororaphis*(ZSB15) maintained at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, India, for commercial biofertilizer production were used for all the experiments. The details of the strains, growth media and conditions are summarized in Table 1.

Table 1. Details of biofertilizer strains used in this study

Particulars	**Strains**			
	Sp7	Pb1	KRB9	ZSB15
Taxonomic affiliation	*Azospirillumbrasilense*	*Bacillusmegateriumvar. phosphaticum*	*Paenibacillusmucilaginosus*	*Pseudomonas chlororaphis*
Plant growth promoting traits	N fixation; production of IAA, cytokinin (Zeatin), gibberellins, ABA, ACC deaminase; polyamine production (Spermidine, spermine, putrescine, and cadaverine); NO production; siderophore production	P solubilization; solubilize zinc oxide, zinc carbonate, K-bentonite production of IAA, gibberellic acid, siderophore, biofilm producer, possess chitinase activity; produce volatiles	K solubilization; solubilize tri-calcium phosphate; N fixation; siderophore production; IAA production	Zn solubilization; solubilize P and K; produce IAA, gibberellin; produce antifungal compounds such as pyrrolnitrin, PCN, PCA, HPR, 2-hydroxyphenazine
Growth medium	Nitrogen-free malic acid medium	Sperber'shydroxy apatite medium	Aleksandrov's medium	Bunt &Rovira medium
Crops tested	Wheat, maize, sorghum, barley, paddy, tomato, garlic, grapevine	Groundnut, sugarbeet, barley, alfalfa, clover, wheatgrass, cicer	Paddy, maize, tomato, groundnut, green gram, apple, Sudan grass	Paddy, maize, tomato
Efficiency	20-40 kg N/ha	483±5mg/L of P was released when supplied with fish bone at the rate of 5g/L	92.2% dissolution of K	47% Zn solubilizing efficiency of zinc oxide
References	[32], [33], [34], [35], [36], [37], [38], [39]	[1], [2], [3], [4]	[5], [6], [7], [8]	[9], [10], [11], [12]

2.2EFFECT OF SEED COAT FORMULATION CONSTITUENTS ON THE GROWTH OF BIOFERTILIZER STRAINS FOR MAIZE SEED COATING UNDER *IN VITRO*

To estimate the growth of biofertilizer strains, Ammonium Mineral Salt (AMS) medium with glucose (1%) was used as the sole carbon source and positive control in this experiment and for the rest, glucose was replaced with dextrin (nutrient supplement for seed coat inoculants) and further supplemented with hydroxypropyl methylcellulose (HPMC, binding agent for seed coating) and assessed the growth pattern of all four strains at different combinations. Dextrin at 1% and HPMC at 5% were supplemented with AMS medium after sterilization. Each biofertilizer strain was grown overnight in Luria Bertani (LB) broth at 30°C and the cells were harvested by centrifugation, washed the pellets twice with phosphate saline buffer, and resuspended in the same buffer. The cell concentration was adjusted to 0.05 OD (approximately 10^8cfu per ml) at 660 nm. From this, 0.5 ml of the culture was inoculated to AMS, AMS + dextrin, AMS + HPMC, and AMS + dextrin + HPMC and incubated at 30°C. The population was enumerated at 24, 48, and 72 h intervals by drop plate method using LB agar medium [19]. Five independent replicates were maintained for each treatment, enumerated the colonies and expressed as log colony-forming units (CFU/ml).

2.3DEVELOPMENT OF NPK AND NPKZN CONSORTIUM FOR SEED COATING

All four biofertilizer strains were grown in their respective growth media at 30°C till the population attains 10^9 cells per ml. The cells were then harvested by centrifugation, washed twice in phosphate buffer (0.1 M, pH 7.0), resuspended in the same buffer, and adjusted the cell concentration to OD660 of 0.05 (10^8CFU/ml). The cell suspensions were then stored at 4°C for further use. For developing NPK consortium, the cell suspension of *Azospirillumbrasilense*(Sp7), Phosphobacteria (Pb1), and Potashbacteria (KRB9) were mixed at an equimolar ratio (1:1:1); and for NPKZn consortium, cell suspension of Zn solubilizing bacteria (ZSB15) also addedat equimolar ratio (1:1:1:1) and used for the entire study.

2.4SEED COATING WITH BIOINOCULANTS

A seed coating mixture containing the bioinoculants (NPK / NPKZn), nutrient supplement (1% dextrin), the polymer (5 % HPMC) and dye (coloring agent) was prepared and used for coating the seed. COH(M)8 maize seeds, a semi-dent, bold grains with medium duration hybrid (85-95 days)were used for this study.Before coating, the maize seeds (COH(M)8) were surface sterilized by soaking the seeds in sodium hypochlorite (3% available chlorine) for 3 minutes and washing with sterile water, followed by soaking in 70% ethanol for 3 minutes, then rinsed thrice with sterile water. The surface sterilized maize seeds were uniformly coated with seed coating mixture for NPK &NPKZn separately and air-dried for 30 min in a laminar airflow chamber and packed in sterile zip lock polythene bags and stored at 4°C for shelf-life assessment.

2.5SEED WEIGHT AND VOLUME

The NPK &NPKZnbioinoculants coated maize seeds were initially checked for the change in physical parameters viz., seed weight and volume on a 100 seed weight basis and compared with the normal seeds.

2.6ESTIMATION OF SHELF LIFE OF BIOINOCULANTS IN THE COATED MAIZE SEEDS

Shelf life was estimated for the survival of individual biofertilizer strain populations survived on the coated maize seeds on the day of coating (0^{th} day) and 3 months after storage by MPN method for *Azospirillumbrasilense* and serial dilution and plating method for

phosphobacteria, potashbacteria and zinc solubilizing bacteria in their respective growth medium.

2.7EFFECT OF SEED COATING OF BIOFERTILIZERS ON MAIZE SEEDLINGS

The effect of NPK and NPKZnbioinoculants on the coated maize seeds were assessed for germination per cent, vigor index, and plant height by roll towel method [20].

2.8STATISTICAL ANALYSIS

All the data were performed one-way or three-way analysis of variance followed by Tukey's honestly significant difference test ($p<0.01$) in R software (Version 4.1.1) (R Core Team, Vienna, Austria) to reveal the statistical significance between the treatments.

3. RESULTS

3.1 EFFECT OF SEED COAT FORMULATION CONSTITUENTS ON THE GROWTH OF BIOFERTILIZER STRAINS FOR MAIZE SEED COATING UNDER *IN VITRO*

Among the different combinations of seed coating constituents viz., (Dextrin, Polymer & Dextrin+ Polymer), Dextrin supplemented AMS recorded a significantly higher growth (12%) in all the biofertilizer strains. AMS supplemented with HPMC declined the bacterial growth. At the same time, AMS + HPMC + dextrin had at par growth as that of AMS control (Fig. 1A). Among the four strains, *Azospirillumbrasilense*(Sp7) showed higher growth compared to other biofertilizer strains, and Phosphobacteria (Pb1) accounted the least growth response to the seed coat components (Fig. 1B). The time-course mean growth of bacterial strains was significantly higher at 72 h after incubation. In contrast, the 24 h and 48 h growth had insignificant differences (Fig. 1C). The time course growth of individual strain as influenced by carbon supplementation was presented in Fig. 1D. The difference due to nutrient supplementation (dextrin and HPMC) was least in case of *Azospirillumbrasilense* (Sp7) and phosphobacteria (Pb1) whereas, the difference was higher for Potash bacteria (KRB9) and Zn solubilizing bacteria (ZSB15) (Fig. 1D). The result also showed that addition of dextrin and polymer (HPMC) in the AMS medium did not cause deleterious effects for the growth of bacterial strains.

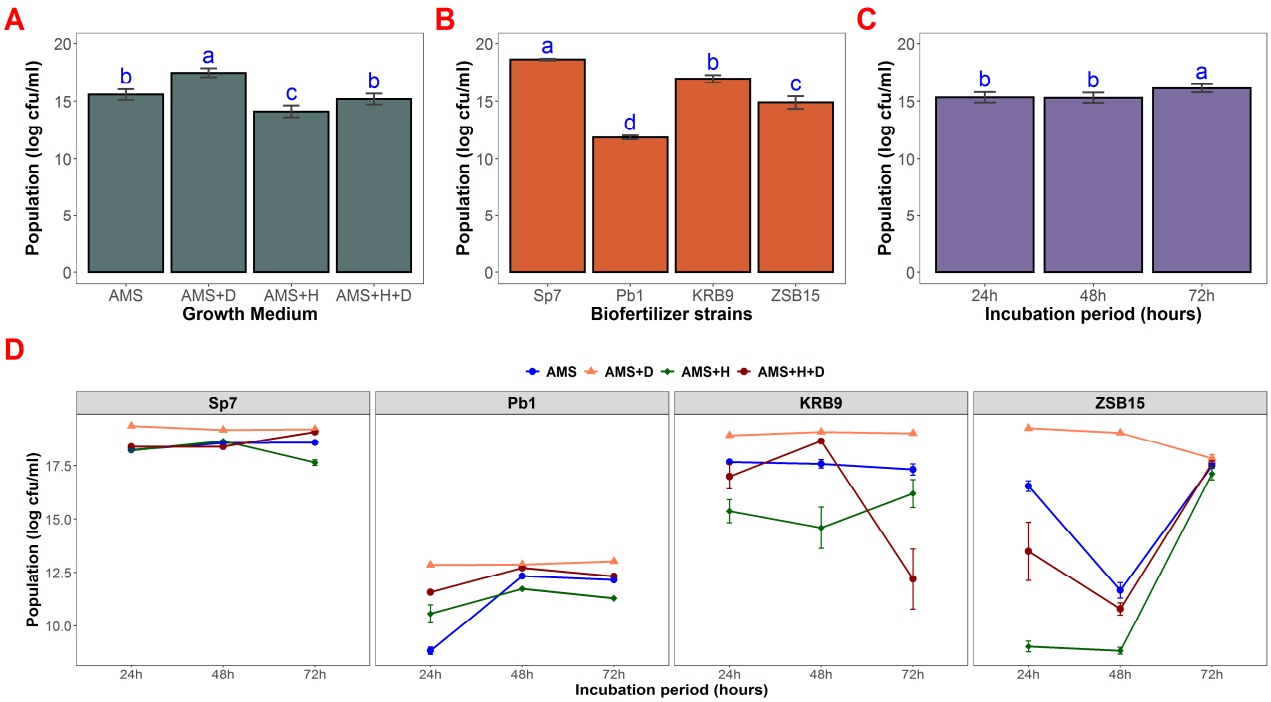

Fig. 1.Impact of dextrin and HPMC on the growth of four different biofertilizer strains used in this experiment

Mean comparison due to the growth medium (A), biofertilizer strains (B), the incubation period (C); and three-way interactions (D). Each panel represents a mean of five replicates, and the error bar indicates standard error. The panels with the same letter are not significantly different as determined by the Tukey test (p≤0.01). AMS – ammonium mineral salt medium; AMD+D – AMS medium amended with dextrin; AMS+H – AMS medium amended with HPMC; AMS+D+H – AMS medium with dextrin and HPMC. SP7 – Azospirillumbrasilense (Sp7); Pb1 – Bacillus megateriumvarphosphaticum (Pb1); KRB9 – Paenibacillusmucilaginosus (KRB9); ZSB15 – Pseudomonas chlororaphis (ZSB15).

3.2ESTIMATION OF SHELF LIFE OF BIOINOCULANTS IN THE COATED MAIZE SEEDS

The survival of each bacterial strain on the maize seed coated with either NPK or NPKZn consortia was assessed onthe 0^{th} day and after 3 months of storage. Irrespective of individual biofertilizer strains, the mean cell load of the maize seed coated with NPK consortium was recorded as 2.88 logCFU per seed. Whereas, the NPKZn consortium coated seed accounted for 3.70 logCFU per seed (Fig. 2A). SP7 > Pb1 ≈KRB9 ≈ ZSB15 was the trend being observed as a strain-level proportion on maize seed (Fig. 2B). A 1.01 log reduction (26%) was observed in the microbial cell load of NPK and NPKZn consortium after 3 months of storage (Fig. 2C). Among the four strains, *Azospirillumbrasilense*(Sp7) had 1.53 and 1.43 log reduction in maize seed coated with NPK and NPKZn consortium followed by Phosphobacteria (1.07 and 0.93 log reductions for NPK and NPKZn coated seeds, respectively). The KRB9 accounted for 1.00 and 0.97 log reductions after 3 months of storage of NPK and NPKZn consortia coated seeds, respectively. The ZSB15 recorded a mean log reduction of 1.28 per seed after 3 months of storage in the NPKZn consortium (Fig. 2D).

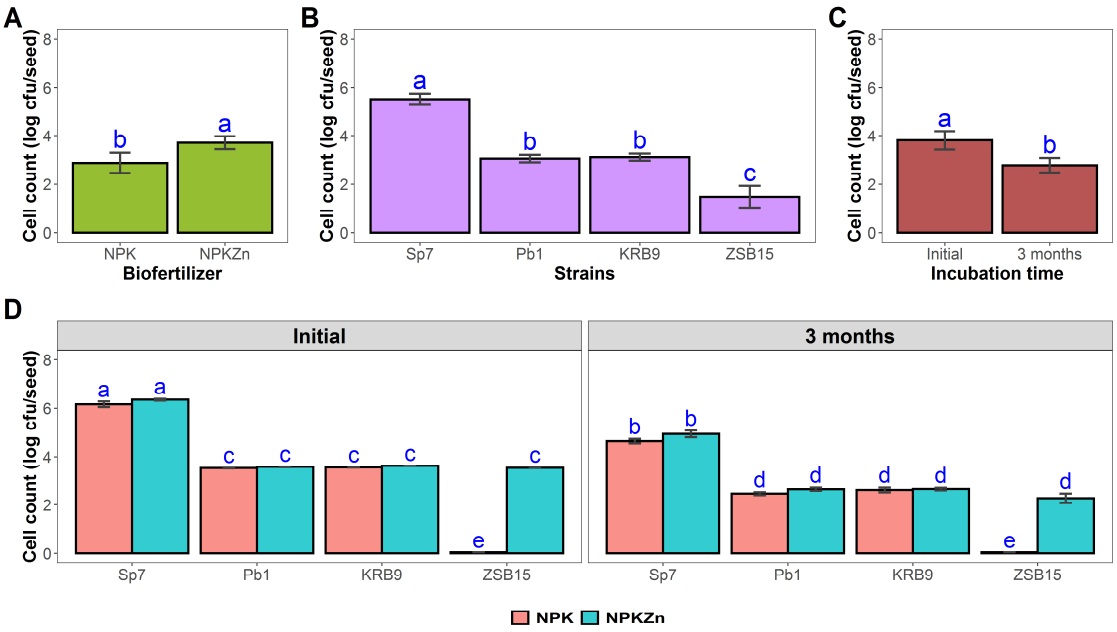

Fig. 2.Proportion of biofertilizer strains on the maize seed coated by NPK- and NPKZn consortia at initial and 3 months after storage

Mean comparison due to the consortium (A), biofertilizer strains (B), the incubation period (C); and three-way interactions (D). Each panel represents a mean of five replicates, and the error bar indicates standard error. The panels with the same letter are not significantly different as determined by the Tukey test (p≤0.01). NPK – consortium consists of Sp7, Pb1, and KRB9; NPKZn – consortium consists of Zp7, Pb1, KRB9, and ZSB15. SP7 – Azospirillumbrasilense (Sp7); Pb1 – Bacillus megaterium var. phosphaticum (Pb1); KRB9 – Paenibacillusmucilaginosus (KRB9); ZSB15 – Pseudomonas chlororaphis (ZSB15). The population of individual strains in consortia was enumerated by plate count method at initial and after storage (3 months).

3.3EFFECT OF SEED COATING OF BIOFERTILIZERS ON MAIZE SEED

The 100 seed weight and volume of coated maize seeds were slightly altered due to seed coating with biofertilizer consortium (Fig. 3). The seed weight was increased by 2.5%, and volume was increased by 8-9% due to seed coating with biofertilizers.

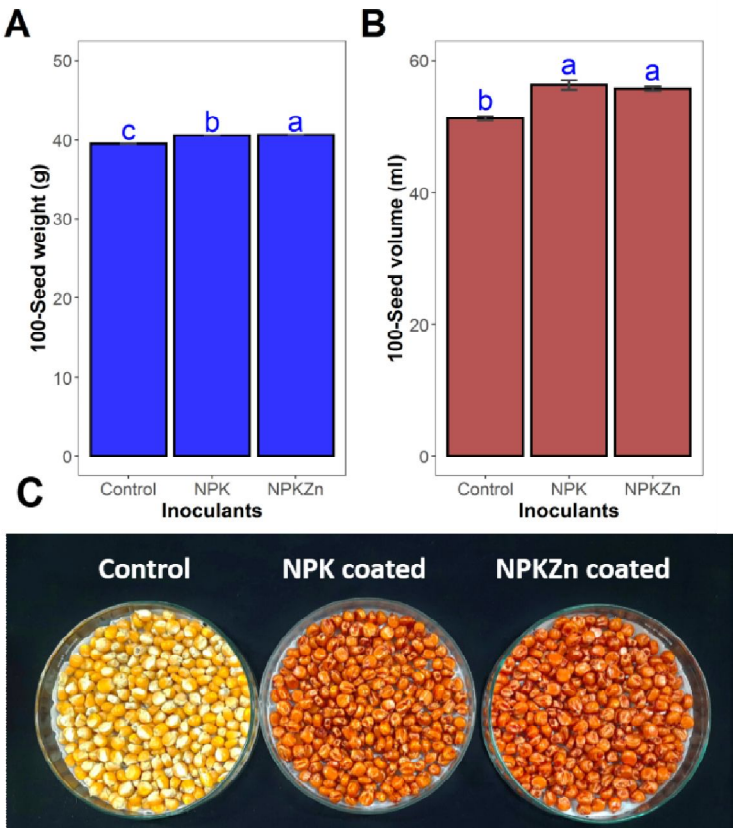

Fig. 3.Changes in seed weight (A) and volume (B) of maize seed due to seed coating with biofertilizer consortium.(C) The appearance of maize seed after seed coating.

Each panel represents a mean of six replicates, and the error bar indicates standard error. The panels with the same letter are not significantly different as determined by the Tukey test (p≤0.01). Control – uninoculated control; NPK – consortium consists of Sp7, Pb1, and KRB9; NPKZn – consortium consists of Zp7, Pb1, KRB9, and ZSB15.

3.4EFFECT OF SEED COATING OF BIOFERTILIZERS ON MAIZE SEED GERMINATION AND GROWTH

The maize seeds coated with NPK and NPKZn consortium showed an insignificant difference in seed germination (Fig. 3A) and seedling vigor (Fig. 3B). However, the maize seedling showed higher growth in terms of plant height due to seed coat formulation of biofertilizers than uninoculated control (Fig. 3C and 3D). The NPK and NPKZn coated maize seeds accounted for 21 and 18% increased plant height of maize than uninoculated control.

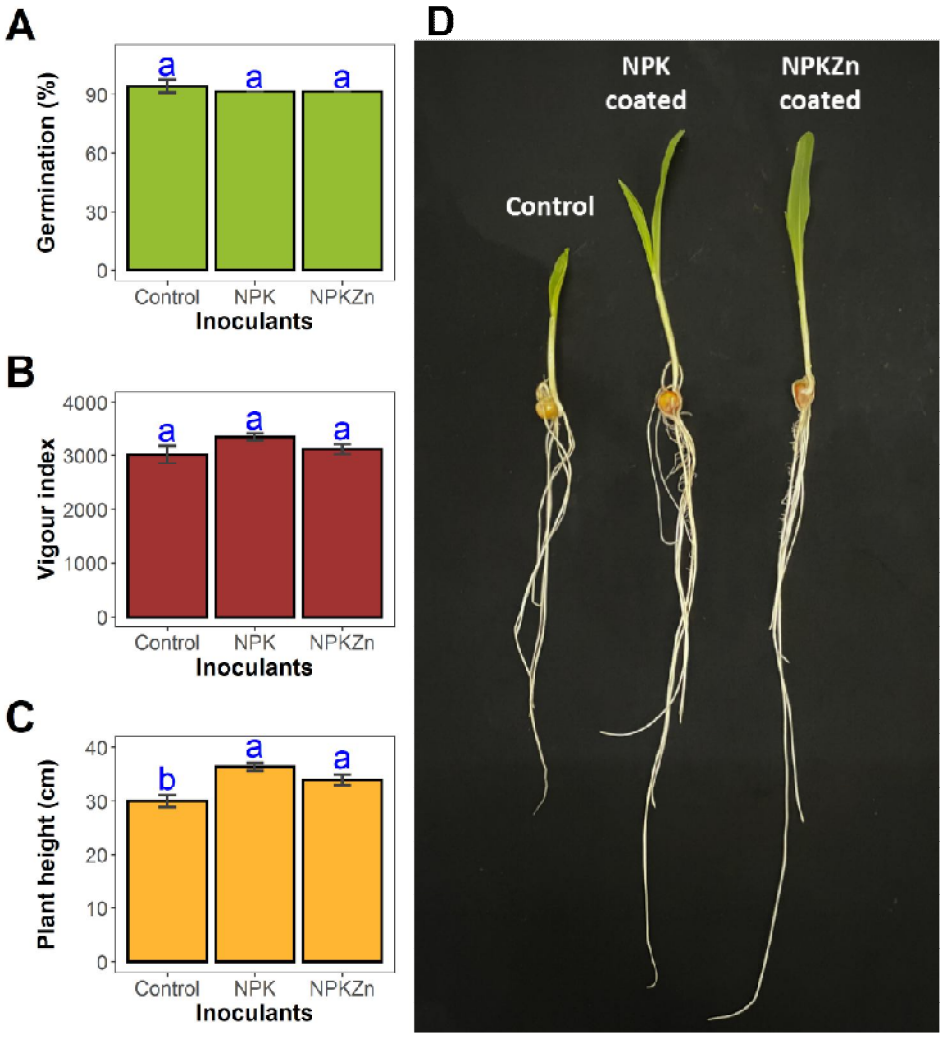

Fig. 4.Impact of biofertilizer seed-coating on germination and seedling growth of maize.(A) Germination per cent; (B) Vigour index; (C) Plant height, and (D) Growth of maize seedlings after 7-days.

Each panel represents a mean of six replicates, and the error bar indicates standard error. The panels with the same letter are not significantly different as determined by the Tukey test (p≤0.01). Control – uninoculated control; NPK – consortium consists of Sp7, Pb1, and KRB9; NPKZn – consortium consists of Sp7, Pb1, KRB9, and ZSB15.

4. DISCUSSION

Biofertilizers can be effectively delivered through seed dressing (instant coating seeds with the formulations just before sowing), seed pelleting (use of fillers and binders to coat the seed with significantly increased weight and volume), or film coating (fillers and binders used but without significant increase of weight and volume) [18]. In the present work, the film coating principle was adopted in maize seed to colonize four important biofertilizers to maximize their benefits effectively. Hydroxy propyl methyl cellulose and dextrin were used as a film-coating molecule, protectant, and binder in the present work. HPMC is an odorless,

transparent, nontoxic, non-ionic, and edible polymer with a linear structure of glucose. HPMC is formed using methyl substitution of the free hydroxyl group of the glucose with the hydroxyl propyl group. This modification improves the cellulose's viscosity, solubility, gelation, and film formation [21]. HPMC is widely used for drug delivery and film coating [22]. However, no attempts were made to use this molecule in microbial inoculant film-coating seeds. Another polymer, dextrin, is a low-molecular-weight mixed polymer containing glucose as a monomer linked with α (1→4) and α (1→6) glycosidic bonds. It is the derivative of starch or glycogen and is widely used as a sticker and coating material in various industries [23]. Recently, dextrin was used as a water-soluble carrier for rhizobium and mycorrhiza inoculant formulation [24].Similarly, Mastouri*et al*. (2010)[25] used cellulose and dextran encapsulation of Trichoderma for tomato seed treatmentwhich showed an increased seedling vigour and reduced lipid peroxides accumulation in seedlings under osmotic stress condition.

The present work attempted the feasibility of combining HPMC and dextrin as ingredients for film-coating biofertilizers. HPMC will form a thin film on the surface, while dextrin acts as a binding agent and protecting agent for the microbial cells for survival upon coated seeds. When these two constituents were supplemented in an AMS medium, all four biofertilizer stains showed no growth inhibition due to HPMC, dextrin, or a combination of both. This result validates that these two polymers could be used effectively for the seed coating of biofertilizers[24].

Shelf-life of microbial-coated seeds is always a critical factor to be considered. The viability of cells on the surface of the seed and the quality of microbial-inoculated seed are two important concerns that need to be addressed for the seed-coat formulation of biofertilizers[26]. Desiccation is the critical factor influencing the survival of microbial inoculants on the seed surface [27]. When freeze-dried microbial inoculants were used to coat the seeds, their survival was enhanced under low relative humidity storage conditions [24, 28]. In the present work, the liquid inoculants of four different biofertilizer strains were mixed at an equimolar ratio and coated on the maize seed using HPMC and dextrin. The shelf-life of each inoculant on the seed was compared with the 0th-day sample. The mean cell count of 10^4 per seed reported at the coating time reduced 1 log (10 cells) after 3 months of storage. This log reduction is trivial, as the minimum of 10^3 cells of each biofertilizer strain is viability present in maize seed after the storage. The film-coated seed stored at low temperature with minimal moisture loss maintained high viable counts of biofertilizer strains. Moreover, the polymers that coat the microbial cells protect the inoculants from various environmental stresses [29].

Among the three seed-coating methods, film-coating is the recently developed method, and it is considered the best method due to less change in size, shape, and weight of the seed [30]. In the present work, HPMC and dextrin-based film coating of the microbial consortium was performed on maize, and no significant changes were observed between coated and uncoated seeds. The seed weight increase of 2.5% and 8% enhanced seed volume without any change in the shape and appearance of maize seed was observed due to film-coating (Fig. 4 of the present work). Hence, film coating microbial inoculants using HPMC and dextrin could be a potential inoculation method with minimal dust-offproblems. Seed coating of biofertilizers aims to improve seed germination, seedling vigor, plant growth, and yield [31]. Several microbial inoculants with different combinations of polymers were investigated for seed coating [29]. The present work assessed the maize seeds coated with two different microbial consortia for their seedling establishment. The results suggested that the germination and seedling vigor of maize seeds were unaffected due to the film coating of microbial inoculants, and the growth of seedlings was enhanced due to microbial

consortium. When the seeds germinated, the inoculated strains effectively colonized the rhizosphere and rhizoplane of maize and promoted plant growth.

4. CONCLUSION

Based on the present investigation, we showed that HPMC and dextrin-based film coating of microbial inoculants on maize seed could be an appropriate and viable technology for the application of biofertilizers. When *Azospirillum*, phosphobacteria, potash-releasing bacteria and zinc solubilizing bacteria as a consortium was coated on maize seed, they survived with a mean population of 10^3 cells of each strain per seed for up to 3 months. The film-coated maize seed did not change its shape, size, weight, and volume compared to the control, and the bioinoculants-coated seed showed increased seedling growth. Hence, this seed coating technology could be a promising approach for the effective delivery of microbial inoculants.

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