

2022-01-20

Effects of Carrier Materials and Storage Temperatures on the Viability and Stability of Three Biofertilizer Inoculants Obtained from Potato (*Solanum tuberosum* L.) Rhizosphere

Aloo, Becky

MDPI

<https://doi.org/10.3390/agriculture12020140>

Provided with love from The Nelson Mandela African Institution of Science and Technology

Article

Effects of Carrier Materials and Storage Temperatures on the Viability and Stability of Three Biofertilizer Inoculants Obtained from Potato (*Solanum tuberosum* L.) Rhizosphere

Becky Nancy Aloo ^{1,*}, Ernest Rashid Mbega ², Billy Amendi Makumba ³ and John Baptist Tumuhairwe ⁴¹ Department of Biological Sciences, University of Eldoret, Eldoret P.O. Box 1125-30100, Kenya² Department of Sustainable Agriculture and Biodiversity Conservation, Nelson Mandela African Institution of Science and Technology, Arusha P.O. Box 447, Tanzania; ernest.mbega@nm-aist.ac.tz³ Department of Biological Sciences, Moi University, Eldoret P.O. Box 3900-30100, Kenya; billymakumba@gmail.com⁴ Department of Agricultural Production, College of Agricultural and Environmental Sciences, Makerere University, Kampala P.O. Box 7062, Uganda; jbtumuhairwe@caes.mak.ac.ug

* Correspondence: baloo@uoeld.ac.ke

Abstract: Biofertilizer technology continues to be derailed by the short shelf life of inoculants. The present study investigated the suitability of wheat-bran (WB), rice-husks (RH), farmyard-manure (FYM), bagasse (BG), and sawdust (SD) in the formulation of potato-derived *Klebsiella grimontii* (MPUS7), *Serratia marcescens* (NGAS9), and *Citrobacter freundii* (LUTT5) under refrigerated (8 °C) and room (25 ± 2 °C) storage. The physicochemical properties of the materials were assessed before sterilization and introduction of the inoculants and assessment of their viability for 8 months. Most of the physicochemical properties of the materials varied significantly ($p < 0.05$). Bagasse supported the maximum growth of MPUS7 (5.331 log CFU g⁻¹) under refrigeration and LUTT5 (4.094 log CFU g⁻¹) under both conditions. Under room storage, the maximum growth of MPUS7 (3.721 log CFU g⁻¹) occurred in WB. Formulations that remained viable under room storage can easily be integrated into existing agricultural distribution systems that lack refrigeration.

Keywords: rhizobacteria; carrier materials; biofertilizer; bioformulations; shelf-life



Citation: Aloo, B.N.; Mbega, E.R.; Makumba, B.A.; Tumuhairwe, J.B. Effects of Carrier Materials and Storage Temperatures on the Viability and Stability of Three Biofertilizer Inoculants Obtained from Potato (*Solanum tuberosum* L.) Rhizosphere.

Agriculture **2022**, *12*, 140.

<https://doi.org/10.3390/agriculture12020140>

agriculture12020140

Academic Editor: Luciano Beneduce

Received: 20 July 2021

Accepted: 7 September 2021

Published: 20 January 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Conventional agricultural practices are a major contributor to environmental pollution, global warming, and climate change [1], especially from the use of artificial fertilizers, which are widely associated with greenhouse gas emissions [2]. As such, attempts to find suitable alternative crop fertilization mechanisms and promote agricultural sustainability are quickly gathering momentum worldwide [3]. It is propounded that soil microbiota are key to the development of sustainable cropping systems [4], and plant rhizospheres have been the center of focus for researchers for decades worldwide.

Various plant-root inhabiting bacteria (rhizobacteria) can promote plant growth through various biochemical processes [5], like the production of phytohormones and siderophores, solubilization of phosphates, and biological nitrogen fixation [6,7]. Such beneficial rhizobacteria can be optimized and formulated into biofertilizers for sustainable crop production using different carrier materials [8].

The concept of biofertilizers is widely researched and there exist several practical applications globally, the utilization of this technology [9]. The use of agricultural wastes as carriers for biofertilizer formulations is one commonly explored option because of their ready availability and cost-effectiveness [10]. However, the survivability and efficiency of rhizobacterial inoculants in biofertilizer formulations are greatly dependent on the choice of carrier materials and storage temperatures [11,12]. As such, designing effective biofertilizers with long shelf lives is the greatest bottleneck for biofertilizer technology [10]. Although

various formulations have been evaluated to date, the shelf-life data of most organic formulations are still grossly inadequate. Research should be intensified to develop stable, functional, and reliable biofertilizer inoculants as tools for sustainable agriculture [13]. The present study aimed to investigate the effects of different agricultural wastes as carrier materials for the formulation of selected potato rhizobacterial inoculants and to evaluate their survivability and stability at the end of a storage period under two different temperature conditions. This will ultimately inform us on the suitable carriers and storage conditions for the studied rhizobacterial inoculants and others for sustainable agricultural systems.

2. Materials and Methods

2.1. Rhizobacterial Strains

Potato tubers and rhizosphere soils were sampled from different parts of Tanzania in June 2018. External rhizobacterial strains were isolated from the soil samples by serial dilution (up to 10^{-3}) using sterile saline water ($\frac{1}{4}$ strength Ringer's solution), plating 1 mL aliquots of the 10^{-3} dilution on Tryptic Soy Agar (TSA), and incubating at 28 ± 2 °C for 48 h [14]. For endophytic isolates, the potato tubers were washed with running tap water and sterilized with 70% (*v/v*) ethanol and 2% (*w/v*) sodium hypochlorite for 30 s and 10 min, respectively, followed by rinsing with sterile distilled water [14] and isolation as described by Aravind et al. [15]. Three rhizobacterial strains, MPUS7, NGAS9, and LUTT5, and identified as *Klebsiella grimontii*, *Serratia marcescens*, and *Citrobacter freundii*, respectively, were selected from among the obtained isolates based on preliminary analyses of their *in vitro* plant growth-promoting abilities and effects on growth of potted potato plants and later characterized [16] for the formulation of biofertilizers. The GenBank accession numbers of these isolates are CP047604, CP047605, and CP047606, respectively.

2.2. Characterization of Carrier Materials and Formulation of Biofertilizers

Approximately 3.5 kg (dry weight) of 5 types of agricultural wastes (bagasse (BG), farmyard manure (FYM), rice husks (RH), sawdust (SD), and wheat bran (WB)) were sourced from locally available agro-industries in Arusha for use as carrier materials. The pH of the materials was determined as described by Arora et al. [17]. The carrier materials were sterilized in the oven for 24 h at 105 °C and the sterilization efficiency was confirmed by plating out 1 g aliquots of samples on TSA and observing no growth after incubating at 28 ± 2 °C for another 24 h. The final (%) moisture contents (MC) were determined on a wet and dry mass basis as expressed in Equation (1).

$$(\%) \text{ Moisture content} = \frac{\text{Mass before drying (g)} - \text{Mass of dry sample (g)}}{\text{Mass of sample before drying (g)}} \times 100\% \quad (1)$$

The water holding capacity (WHC) of each material was assessed using the Keen-Raczowski cup method following the procedures described by Joshi and Setty [18] and Equation (2).

$$(\%) \text{ WHC} = \frac{\text{Mass of saturated and drained sample (g)} - \text{Mass of dry sample (g)}}{\text{Mass of dry sample (g)}} \times 100\% \quad (2)$$

The electrical conductivity (EC) of carriers was determined using the saturated paste method (Chi and Wang, 2010) while the organic carbon (OC) and organic matter (OM) contents were evaluated using the potassium dichromate wet digestion method [19]. The N contents were quantified using the micro-Kjeldahl method [20] while the Mehlich III extraction method [21] was used to extract exchangeable Zn and P from materials. The ammonium acetate extraction method [22] and the 1, 10-phenanthroline complex method [23] were used to extract exchangeable K and Fe from the carriers, respectively. To estimate the quantities of P, K, Zn, and Fe in the respective carrier extracts, their optical densities were obtained spectrophotometrically at A690, A799, A399, and A510, respectively, using a multi-mode reader (Synergy HTX-Biotek). The quantities of P, K, and Zn in mg kg^{-1}

were calculated from standard curves prepared from standard solutions of KH_2PO_4 , KCl , ZnSO_4 and $\text{Fe}(\text{NH}_4)_2 (\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, respectively.

The rhizobacterial strains were multiplied in TSB medium by inoculating and incubating at $28 \pm 2 \text{ }^\circ\text{C}$ in a shaking incubator (180 rpm) for 24 h for them to attain log phase growth with a cell load of about $1.8 \times 10^6 \text{ CFU mL}^{-1}$ [24,25]. The carriers were used to formulate biofertilizers for the 3 rhizobacterial inoculants (*K. grimontii* MPUS7, *S. marcescens* NGAS9, and *C. freundii* LUTT5) following the procedure described by Abd El-Fattah [24]. For each of the 3 inoculants, two sets of each sterilized carrier material (~20 g) were packed in sterile high-density clear plastic containers (40 × 30 × 15 cm) in triplicates for storage at $8 \text{ }^\circ\text{C}$ and $25 \pm 2 \text{ }^\circ\text{C}$, respectively, for the monthly evaluations of inoculant viability. A summary of the design used to formulate these biofertilizers is shown in Table 1.

Table 1. A summary of the design used to formulate biofertilizers out of the rhizobacterial inoculants.

Carrier Material		BG ¹	FYM ²	SD ³	WB ⁴	RH ⁵	Purpose
<i>Klebsiella grimontii</i> MPUS7	Set 1	20 g	20 g	20 g	20 g	20 g	8 °C storage
	Set 2	20 g	20 g	20 g	20 g	20 g	25 °C storage
<i>Serratia marcescens</i> NGAS9	Set 1	20 g	20 g	20 g	20 g	20 g	8 °C storage
	Set 2	20 g	20 g	20 g	20 g	20 g	25 °C storage
<i>Citrobacter freundii</i> LUTT5	Set 1	20 g	20 g	20 g	20 g	20 g	8 °C storage
	Set 2	20 g	20 g	20 g	20 g	20 g	25 °C storage

¹ Bagasse, ² Farmyard manure, ³ Sawdust, ⁴ Wheat bran, ⁵ Rice husks.

The preparations were thoroughly mixed in sterile Petri-dishes (90 mm) using sterile glass rods to ensure uniformity in distribution and absorption of the liquid cultures into the carriers and left to cure under sterile conditions in the laminar flow hood for 24 h, repackaged in the sterile plastic containers and sealed aseptically. The 1st sets of each bio-formulation were stored at $8 \text{ }^\circ\text{C}$ and the 2nd sets at room temperature ($25 \pm 2 \text{ }^\circ\text{C}$) for the purpose of determining the conducive storage temperatures. The total number of formulations was thus, 30. During the experiment, aliquots of non-inoculated carrier materials were maintained under the same incubation conditions to check the maintenance of axenic conditions. The biofertilizers packaged for storage are displayed in Figure 1.



Figure 1. Formulated biofertilizers packaged in different carrier materials for storage and monthly evaluations.

2.3. Laboratory Evaluation of the Viability of the Biofertilizer Formulations

Periodical samples were taken aseptically from each of the 30 formulations for 8 consecutive months for evaluation of the stability and viability of the rhizobacterial inoculants. Sampling was done by scooping about 1 g of each formulation using a sterile spatula and suspending in 9 mL of sterile distilled water, followed by vigorous mixing and filtering in Whatman (No. filter papers. The number of viable cells g^{-1} of each formulation was determined using the plate count technique ($28 \text{ }^\circ\text{C}$; 24 h) using the prepared filtrates [24,25].

2.4. Statistical Analyses

All statistical analyses were performed using the XLSTAT (Version 2.3, Adinsoft) at a 95% level of confidence. The Shapiro–Wilk test was used to test for normality of

data and multiple comparisons of variances were performed using Multivariate Analysis of Variance (MANOVA). Variables with significantly different means were subjected to posthoc analysis using Tukey's Honest Significant Difference (HSD) test. A t-test for paired samples was used to evaluate the viability of the biofertilizer formulations in the different carrier materials under room and refrigerated storage.

3. Results and Discussions

3.1. The Physicochemical Characteristics of the Carrier Materials

The physicochemical properties of the carrier materials that were used in the formulation of biofertilizers are portrayed in Table 2. According to Gade et al. [26], these properties can largely affect inoculant survival and viability. The results showed that the materials were moderately acidic (pH 4.73 ± 0.13 to 5.4 ± 0.21) except for FYM which was slightly alkaline (pH 8.38 ± 0.24). Such pH, which is generally near-neutral, is known to support large inoculant populations and maintain their viability [27–29] and maintain their viability.

Table 2. Characteristics of the carrier materials used in the formulation of biofertilizers for in vitro and field evaluations.

	Sawdust	Wheat Bran	FYM	Bagasse	Rice Husks	Average	<i>p</i> Value
pH	4.73 ± 0.13^b	4.63 ± 1.07^b	8.38 ± 0.24^a	4.35 ± 0.19^b	5.37 ± 0.21^b	5.49 ± 1.59	0.000 *
EC ($\mu\text{S cm}^{-1}$)	0.116 ± 0.027^c	1.174 ± 0.634^{ab}	1.985 ± 0.349^a	0.194 ± 0.062^c	0.89 ± 0.026^{bc}	0.993 ± 0.853	0.000 *
WHC (%)	388 ± 22^b	155 ± 28^c	101 ± 7^c	568 ± 90^a	62 ± 46^c	255 ± 203	0.000 *
MC (%)	17.1 ± 0.6^a	10.3 ± 0.5^b	15.8 ± 1.4^a	8.6 ± 1.5^b	8.2 ± 0.3^b	11.9 ± 3.9	0.000 *
N (%)	0.100 ± 0.03	0.060 ± 0.04	0.130 ± 0.01	0.090 ± 0.08	0.160 ± 0.01	0.110 ± 0.05	0.154
OC (%)	1.84 ± 0.17	0.70 ± 0.51	1.45 ± 0.14	1.07 ± 0.92	1.31 ± 0.37	1.27 ± 0.58	0.154
OM (%)	3.17 ± 0.28	1.20 ± 0.88	2.50 ± 0.24	1.85 ± 1.58	2.25 ± 0.63	2.20 ± 1.00	0.152
P (mg kg^{-1})	248.7 ± 103.7^b	323.6 ± 109.2^b	1354.6 ± 68.9^a	183.3 ± 29.9^b	270.8 ± 64.4^b	476.2 ± 462.0	0.000 *
K (mg kg^{-1})	9.26 ± 5.16	9.06 ± 1.44	11.70 ± 0.96	6.24 ± 0.52	7.49 ± 0.96	8.75 ± 2.99	0.225
Zn (mg kg^{-1})	221.3 ± 136.5^{ab}	203.8 ± 69.33^{ab}	442.6 ± 30.99^a	87.4 ± 33.22^b	266.1 ± 192.4^{ab}	244.2 ± 152.3	0.034 *
Fe (mg kg^{-1})	0.99 ± 0.29^b	1.24 ± 0.49^b	3.68 ± 1.62^a	0.72 ± 0.14^b	0.84 ± 0.33^b	1.49 ± 1.32	0.005 *

Values are means of three replicates \pm standard deviation. Means with similar letter superscripts within the same row are not significantly different (Tukey's HSD; $p > 0.05$). * Significantly different at $p < 0.05$.

The average EC of carrier materials ranged from ~ 0.116 to 1.985 dS cm^{-1} . The average EC of RH and BG were 0.89 ± 0.23 and 0.19 ± 0.06 dS m^{-1} , respectively. However, in a study by Khavazi et al. [30] these two carriers portrayed a relatively higher average EC of ~ 4.20 and 1.62 dS m^{-1} , respectively. Similar findings in the range of 3.89 to 4.03 dS m^{-1} have also been reported by Abdel Nabi et al., (2016). The EC of carrier materials depicts the concentration of soluble salts which can definitely influence the activities and survivability of inoculants.

The WHC of the carrier materials ranged from about 62 to 387%. The average WHC of WB, FYM and RH were all $<150\%$ while BG portrayed the greatest average WHC of $568 \pm 90\%$ followed distantly by SD at $388 \pm 22\%$. High WHC ($>50\%$) is a desirable feature in carrier materials to support proper bacterial growth and multiplication [28,29,31]. In the present study, the WHC of the carrier materials ranged from 62 to 387%, suggesting their potential as carriers for biofertilizer production. This is because high WHC favors the enzymatic processes involved in the degradation of the organic materials that provide important nutrients for bacteria [32]. These present findings are also comparable to previous reports [26,27,30]. Sawdust had the 2nd highest average WHC ($388 \pm 22\%$) after BG ($568 \pm 90\%$), agreeing with previous reports about its suitability for biofertilizer formulation [17,25].

The MC of the carrier materials ranged from 8.2 to 17.1%. Additionally, FYM and SD had significantly higher MC averages of $15.8 \pm 1.4\%$ and $17.1 \pm 0.6\%$, respectively, than WB, SD, and BG. Arora et al. [27], while studying different organic carrier materials,

similarly reported an average MC of 16.8% but BG had a lower average MC of 6.8%. The MC of carrier materials can exert a great effect on inoculant survival and longevity [11,33]. Generally, dry formulations with low MC can extend microbial survival for longer periods and at higher temperatures, subsequently reducing marketing and maintenance costs since refrigeration is not required [34]. This is probably because the inoculants can remain inactive and resistant to environmental stresses and insensitive to contamination in carriers with low MC [33].

Generally, the carrier materials were rich in C, N, K, and micronutrients (Fe and Zn) which makes them suitable for microbial growth because these macro and micro-nutrients are required for carbohydrate metabolism [28], protein synthesis, enzyme activation and different growth processes of microbes [35]. For N, OC and OM, averages of $0.100 \pm 0.1\%$, $1.3 \pm 0.6\%$ and $2.2 \pm 1\%$ were recorded, respectively. Farmyard manure seemed to contain more macro and micronutrients than the other carrier materials but its Zn content was not significantly higher ($p > 0.05$) than the rest except for BG. For N, OC and OM, averages of $0.100 \pm 0.1\%$, $1.3 \pm 0.6\%$ and $2.2 \pm 1\%$ were recorded, respectively, in FYM. Previous studies also indicate that FYM contains 0.500 to 1.000% N [36,37], while RH and BG, averagely 0.400% [30]. The SD in this study also contained substantially high OC/OM which makes it a desirable carrier material [17]. Generally, carrier materials with high OM content can increase bacterial survival and enhance the efficacy of bio-formulations [38], by supporting proper inoculant growth and multiplication [31].

3.2. Laboratory Evaluation of the Viability and Stability of the Formulated Biofertilizers

3.2.1. Laboratory Evaluation of the Shelf Life of the Formulated Biofertilizer

The present study also investigated the viability and stability of rhizobacterial biofertilizers that were formulated from the five different carrier materials (WB, SD, FYM, RH, and BG) for 8 months (February–September 2019) under refrigeration ($8\text{ }^{\circ}\text{C}$) and room storage ($25 \pm 2\text{ }^{\circ}\text{C}$). The results are displayed in Figure 2a–f. The determination of shelf lives of formulations is a crucial step during the development of biofertilizers [8,39], and the prolonged survival of inoculants during storage is a desirable feature that can enhance the industrial applicability of biofertilizer formulations [8].

The maximum inoculant population ($5.331\text{ log CFU g}^{-1}$) of *K. grimontii* MPUS7 was achieved in BG after 4 months of storage under refrigeration but the FYM formulation also maintained relatively high *K. grimontii* MPUS7 populations for most of the storage period under the same conditions (Figure 2a), a strong indication of its potential suitability as a carrier for this inoculant under refrigeration, probably due to its lower average MC ($8.62 \pm 1.45\%$) which may make inoculants to remain relatively inactive and resistant to environmental stresses [33]. The WB formulation did not sufficiently maintain high *K. grimontii* MPUS7 populations under refrigeration and by the 7th month of storage, no viable cells were detected from this formulation. Nevertheless, carriers that can maintain inoculants for at least 2–3 months are still suitable for commercial purposes [39,40]. Interestingly, under room storage, the WB formulation maintained the highest inoculant populations for most of the storage period except from the 7th month onwards where the inoculant population dropped to $1.5\text{ log CFU g}^{-1}$ below levels observed in the other formulations (Figure 2b). Although the storage of biofertilizers is mostly reported to be better under refrigeration than room conditions [26,41–43], the present results show that WB could sufficiently maintain *K. grimontii* MPUS7 under room storage, which may allow for its integration in the existing agricultural distribution systems that lack refrigeration facilities [44].

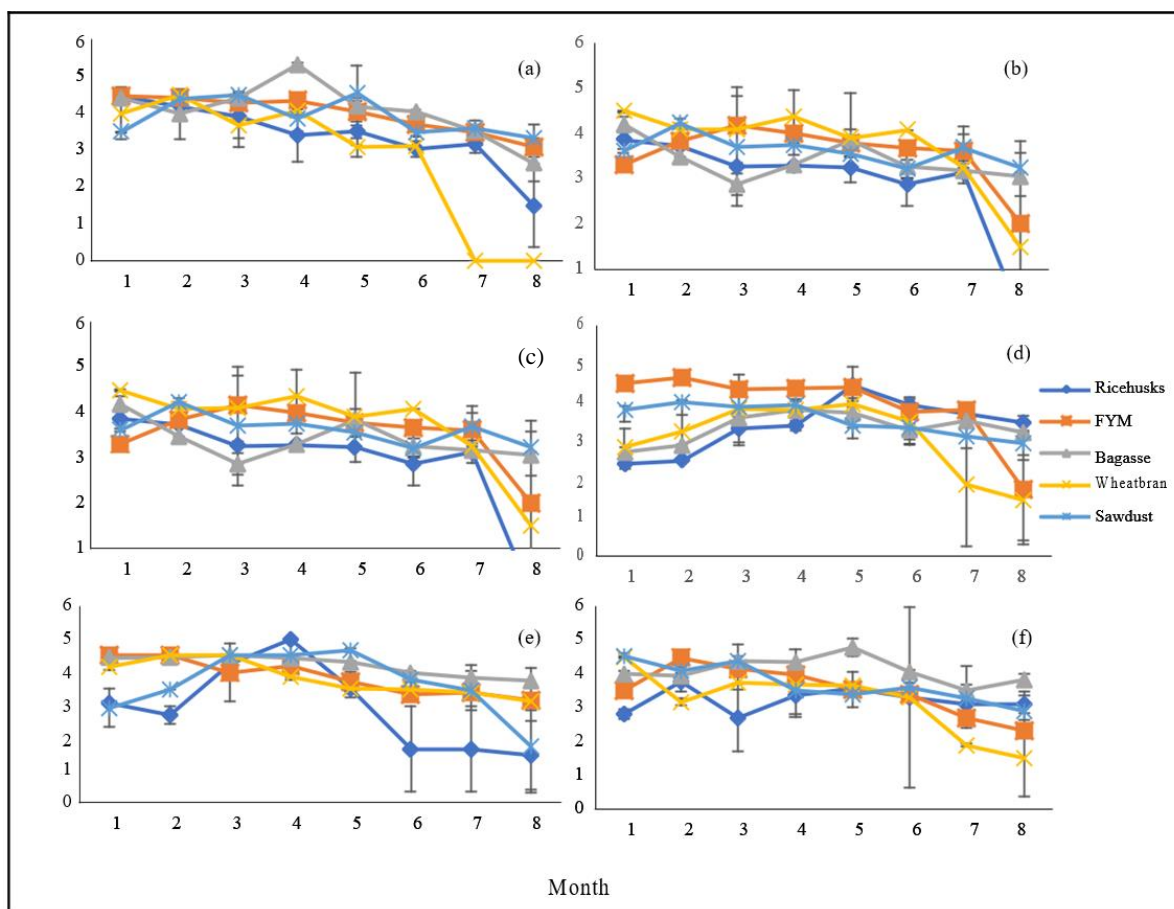


Figure 2. The 8-month viability of the formulated biofertilizers under room and refrigerated conditions in different carrier materials. The graphs represent *K. grimontii* MPUS7 under refrigerated (a) and room (b) storage, *S. marcescens* NGAS9 under refrigerated (c) and room (d) storage, and *C. freundii* LUTT5 under refrigerated (e) and room (f) storage in the different carrier materials. The point values are means of three replicates \pm standard deviation. The y-axes represent the Log CFU g⁻¹ of the biofertilizer formulations.

Similar observations were made for FYM formulation which also maintained relatively high inoculant populations for most of the storage period and by the 8th month, the population of inoculants was still 2.0 log CFU g⁻¹. Under these conditions, the inoculant populations in RH and WB formulations dropped to 0.0 CFU g⁻¹ and 1.5 log CFU g⁻¹, respectively, by the 8th month of storage. Farmyard manure, WB, BG, and SD formulations could all maintain high *S. marcescens* NGAS9 populations throughout the study period under refrigerated conditions (Figure 2c), demonstrating their suitability in supporting the growth and multiplication of this inoculant. Although previous studies have demonstrated RH as a good carrier material for inoculant formulation [25,31,45], such results were not replicated in the present study for *S. marcescens* NGAS9 under refrigeration (Figure 2c) probably due to physiological differences among inoculants [46].

Under room storage, the formulations could all maintain sufficient *S. marcescens* NGAS9 populations during the entire storage period (Figure 2d), suggesting their suitability for the formulation of this inoculant and storage under room conditions which can allow for its integration into existing agricultural distribution channels that lack refrigeration facilities [44]. The suitability of these materials as carriers for other inoculants under room storage has also been demonstrated for other inoculants [27,47]. By the 8th month of room storage, only FYM and WB formulations had low inoculant populations (<1.5 log CFU g⁻¹).

The BG formulation maintained consistently high *C. freundii* LUTT5 populations throughout the study period under refrigeration (Figure 2e).

The compositional analysis of BG has in earlier studies revealed that it is equipped with monosaccharides, hemicellulose, and amino acids that together make it a nutritionally rich material for microbial growth [48]. In a similar study, when BG formulations were stored at 4 °C for 6 months, the survival of inoculants was high and densities of 10^9 cells g^{-1} were maintained [30]. Under room storage, *C. freundii* LUTT5 populations did not vary widely in the formulations (Figure 2f). Nevertheless, the BG formulation proved to be the best at maintaining high *C. freundii* LUTT5 populations and by the 8th month of storage, this formulation still had an average of 3.8 log CFU g^{-1} . Bagasse has also been reported to maintain high numbers of *P. fluorescens* (9.3 log CFU g^{-1}) and *R. leguminosarum* (8.9 log CFU g^{-1}) after 6 months of room storage (25 ± 2 °C) [27]. The shelf life of inoculants depends on several factors including the production technology, carrier material, storage conditions, and packaging material [49]. In the present study, the shelf lives of the formulated biofertilizers were only established in terms of the storage conditions and carrier materials. The viability of inoculums in formulations for a sufficient period is important for the commercialization and applicability of biofertilizers [27]. However, it should be noted that inoculant survival in carriers is not only vital during storage but also after introduction in the soil where they have to compete with well-established indigenous soil microbes [40].

3.2.2. Comparative Effects of Storage Temperatures on the Viability of Biofertilizer Formulations

The present study evaluated the viability of each of the biofertilizer formulations in different carrier materials under room (25 ± 2 °C) and refrigerated (8 °C) conditions using the t-test for paired samples/two-tailed tests. The results are portrayed in Figure 3a–o. Temperature is one of the factors that affect the longevity and survival of inoculants and should be evaluated to optimize storage conditions for long-term inoculant survival [11,33]. Significant differences ($p < 0.05$) were noted between the number of viable cells under room (25 ± 2 °C) and refrigerated (8 °C) conditions for the WB formulations of all 3 biofertilizer inoculants (Figure 3a–c). In a study by Sandikar and Awasthi [42], biofertilizer inoculants also showed a maximum population under refrigeration (8 °C). The prolonged shelf life of inoculants under refrigeration has previously been linked to the reduction of metabolic activities and physiological activities [26,41]. While investigating the shelf lives of *Rhizobium* carrier-based biofertilizers under different storage temperatures, refrigeration (8 °C) was also demonstrated to be more suitable for inoculant viability probably because of lowered MC at higher temperatures [43].

Except for *K. grimontii* MPUS7, the viability of the tested inoculants in the RH formulations although not significant, seemed to be better under refrigeration than room storage (Figure 3j–l). Similar to the observations made for FYM formulations, the viability of inoculants in the BG formulations also seemed to be better under refrigerated conditions (Figure 3k–o). For the SD formulations, the number of viable cells of the three inoculants was also greater under refrigerated than room storage (Figure 3d–f). However, significant differences in 14 these numbers between the two conditions were only evident for *S. marcescens* NGAS9 ($p = 0.001$) (Figure 3e). Similarly, for the FYM formulations, the number of viable cells of all three biofertilizer inoculants was higher under refrigerated conditions but this was only significant for *C. freundii* LUTT5 ($p = 0.020$) (Figure 3g–i). Due to the biological nature of inocula, the survival of formulated cells at room temperature for longer storage is a persistent problem probably due to their growth and metabolic activities which lead to pH changes, death, and loss of viability [50].

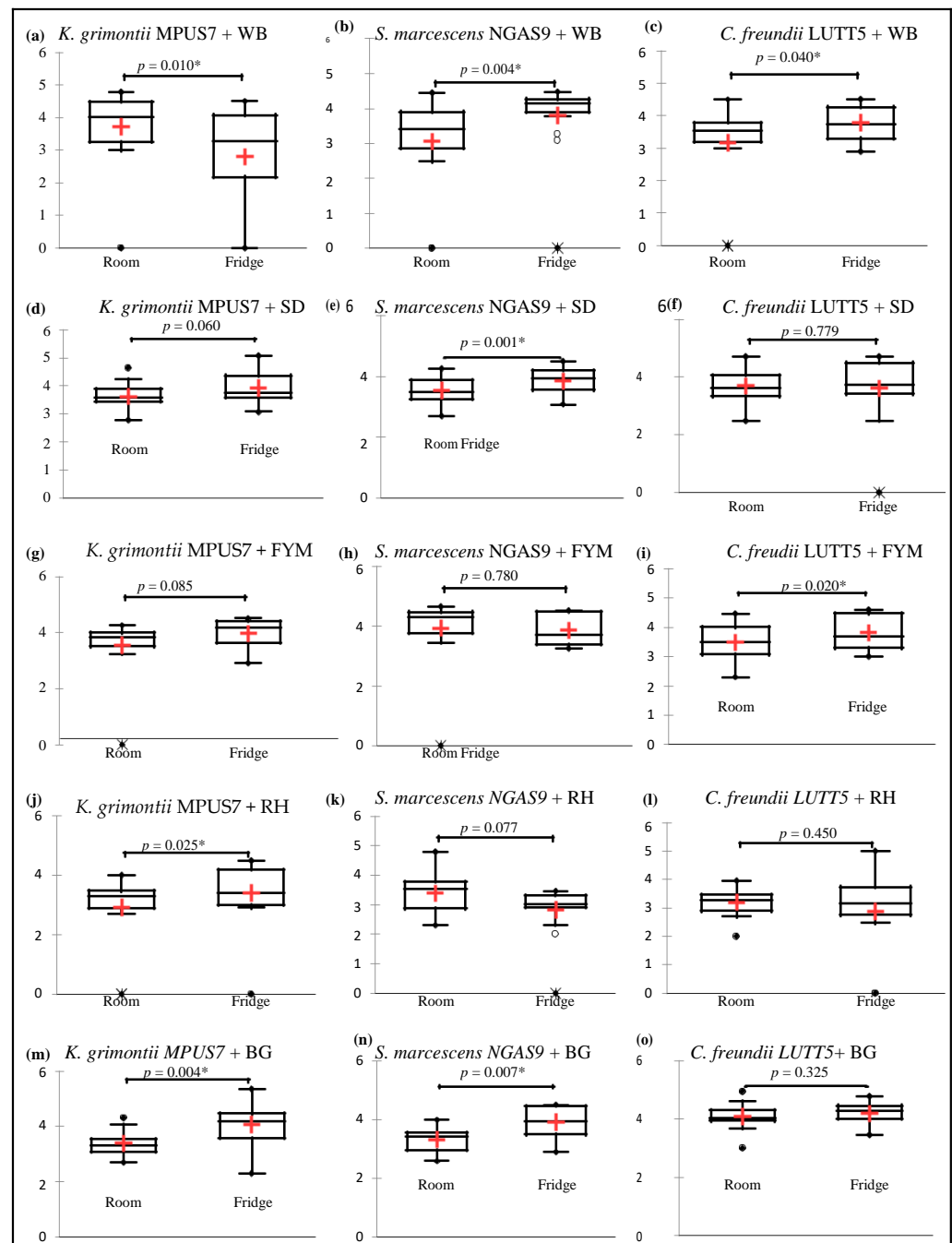


Figure 3. Comparative evaluation of the stability and viability of *K. grimontii* MPUS7, *S. marcescens* NGAS9 and *C. freundii* LUTT5, formulated in wheat bran (WB), sawdust (SD) and farmyard manure (FYM), rice husks (RH) and bagasse (BG) under room and refrigerated storage. Values are means of three replicates \pm standard deviation. The y-axes represent the Log CFU g^{-1} of the biofertilizer formulations. * Significantly different at $p < 0.05$. Significant differences in inoculant means between room and refrigerated storage were obtained for *K. grimontii* MPUS7 + WB (a), *S. marcescens* NGAS9 + WB (b), *C. freundii* LUTT5 + WB (c), *S. marcescens* NGAS9 + SD (e), *C. freundii* LUTT5 + FYM (i), *K. grimontii* MPUS7 + RH (j), *K. grimontii* MPUS7 + BG (m), and *S. marcescens* NGAS9 + BG (n). No significant differences were observed for the mean inoculant numbers between room and refrigerated storage for *K. grimontii* MPUS7 + SD (d), *C. freundii* LUTT5 + SD (f), *K. grimontii* MPUS7 + FYM (g), *S. marcescens* NGAS9 + FYM (h), *S. marcescens* NGAS9 + RH (k), *C. freundii* LUTT5 + RH (l), and *C. freundii* LUTT5 + BG (o).

Unlike *S. marcescens* NGAS9 (Figure 3b) and *C. freundii* LUTT5 (Figure 3c), the number of viable cells of *K. grimontii* MPUS7 in the WB formulation was significantly higher under room storage ($3.7 \pm 1.1 \log \text{CFU g}^{-1}$) than refrigerated conditions ($2.8 \pm 1.7 \log \text{CFU g}^{-1}$) (Figure 3a). Similar observations were also made for *S. marcescens* in FYM (Figure 3h) and RH (Figure 3k). These results demonstrate that these formulations can easily be integrated into existing agricultural distribution systems that lack refrigeration facilities [44].

3.2.3. Comparative Effects of Carrier Materials on the Viability of Biofertilizer Formulations

The viability of the biofertilizer inoculants in different carrier materials under room and refrigerated conditions were also evaluated using the students' t-test for independent variables. The comparative effects of the studied carrier materials on the stability of the inoculants are shown in Table 3. Under room conditions, the WB, SD, FYM, and RH formulations almost equally supported the viability and stability of the three inoculants. However, for the BG formulations, significant differences ($p \leq 0.0001$) were noted for the population of the three inoculants and this formulation seemed to support the growth of *C. freundii* LUTT5 better than *K. grimontii* MPUS7 and *S. marcescens* NGAS9 under the same storage conditions. This may be related to the physiological differences between these rhizobacterial species coupled with carrier-based factors [11,26,30,51]. Under refrigeration, however, significant differences of inoculant populations were only noted for the WB formulations ($p = 0.038$) while the rest of the carrier-based formulations equally supported and maintained the viability of all three biofertilizer inoculants during the experimental period. These results are probably due to the differences in the physicochemical properties of the carrier materials [11,30,51]. Despite having the greatest nutrient contents among the studied carriers, FYM did not significantly influence the stability of inoculants probably because it was also more alkaline (pH 8.38) and had a higher MC (15.8%) since these properties are not suitable for inoculant survival and viability [28,29,34].

Table 3. Comparative evaluation of the viability of *K. grimontii* MPUS7, *S. marcescens* NGAS9 and *C. freundii* LUTT5 ($\log \text{CFU g}^{-1}$) in different carrier materials under room and refrigerated storage.

Storage Conditions	<i>K. grimontii</i> MPUS7	<i>S. marcescens</i> NGAS9	<i>C. freundii</i> LUTT5	<i>p</i> Value
Room Conditions ($25 \pm 2^\circ\text{C}$)				
Wheat Bran	3.721 ± 1.107^a	3.055 ± 1.265^a	3.168 ± 1.267^a	0.285
Sawdust	3.626 ± 0.464^a	3.544 ± 0.414^a	3.697 ± 0.599^a	0.702
Farm Yard Manure	3.548 ± 0.962^a	3.935 ± 1.077^a	3.495 ± 0.694^a	0.372
Rice Husks	2.921 ± 1.159^a	3.385 ± 0.670^a	3.202 ± 0.463^a	0.302
Bagasse	3.398 ± 0.418^b	3.351 ± 0.404^b	4.094 ± 0.424^a	<0.0001
Refrigerated Conditions (8°C)				
Wheat Bran	2.799 ± 1.694^b	3.806 ± 1.056^a	3.792 ± 0.515^a	0.038
Sawdust	3.934 ± 0.506^a	3.850 ± 0.478^a	3.607 ± 1.125^a	0.482
Farm Yard Manure	3.977 ± 0.509^a	3.868 ± 0.512^a	3.825 ± 0.561^a	0.702
Rice Husks	3.401 ± 1.039^a	2.829 ± 0.826^a	2.855 ± 1.553^a	0.351
Bagasse	4.071 ± 0.755^a	3.927 ± 0.516^a	4.187 ± 0.353^a	0.456

Values are means of three replicates \pm standard deviation of the mean. Values with the same letter superscripts in the same row are not significantly different ($p > 0.05$; t-test for paired samples).

The choice of carrier material is one of the factors that affects the efficacy of biofertilizer formulations [11,30,51], and can greatly enhance the shelf life and performance of microbial inoculants [12,35,40]. When selecting carrier materials for biofertilizer formulation, it is necessary to use those that can support high cell numbers to achieve longer shelf lives and increase their usability. The present study investigated on various agricultural wastes as carriers for biofertilizer formulation because they are rich sources of nutrients for microbial growth and are readily available [10]. This not only makes them cheap but also contributes to the reduction of pollution by putting the agricultural wastes into

reuse [38]. These materials were also selected because of their solid nature which makes them compatible with the application technologies for conventional fertilizers.

The overall observations showed that the numbers of viable cells significantly declined in all bio-formulations during the incubation period, similar to previous observations [52]. Such declines may be attributed to the depletion of nutrients, moisture, and autolysis of cells [10,11,41]. At the moment, various efforts in the biofertilizer technology are aimed at selecting carriers and developing novel formulations that can support high inoculant populations and plant growth [25,39], and this study provides the baseline regarding the survivability of various inoculants in organic carrier materials.

4. Conclusions

Except for OC, OM and K quantities, the carrier materials had significantly different physicochemical properties which can greatly influence their suitability as carriers for inoculants. Most of the rhizobacterial formulations could be stored for up to 6 months under both room and refrigerated conditions. Nevertheless, the viability and stability of the inoculants in the formulations seemed better under refrigeration. The carrier materials and storage temperatures both influenced inoculant stability and viability. This study sets a good platform for further explorations into the potential of the formulations for the biofertilization of potatoes and other crops under controlled and field experiments.

Author Contributions: Conceptualization, B.N.A. and E.R.M.; methodology, B.A.M. and J.B.T.; investigation and data collection, B.N.A.; writing—original draft preparation, B.N.A.; writing—review and editing, E.R.M., B.A.M. and J.B.T.; supervision, E.R.M., B.A.M. and J.B.T.; project administration, B.N.A.; funding acquisition, B.N.A. and J.B.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by DAAD, grant number 91671357 and the APC was funded by L’Oreal-UNESCO Foundation For Women in Science, and the African -German Network of Excellence in Science (AGNES), with generous funding from the Federal Ministry of Education and Research (BMBF), and the Alexander von Humboldt Foundation (AvH).

Institutional Review Board Statement: Not Applicable.

Data Availability Statement: All data relating to this work is presented within the article.

Acknowledgments: The authors acknowledge University of Eldoret for providing financial support to the lead author to conduct the study. The authors also acknowledge Dalmas Mnyang’ali for the technical support offered during the laboratory experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bellarby, J.; Stirling, C.; Vetter, S.H.; Kassie, M.; Kanampiu, F.; Sonder, K.; Smith, P.; Hillier, J. Identifying Secure and Low Carbon Food Production Practices: A Case Study in Kenya and Ethiopia. *Agric. Ecosyst. Environ.* **2014**, *197*, 137–146. [[CrossRef](#)]
2. Di Benedetto, N.A.; Corbo, M.R.; Campaniello, D.; Cataldi, M.P.; Bevilacqua, A.; Sinigaglia, M.; Flagella, Z. The Role of Plant Growth Promoting Bacteria in Improving Nitrogen Use Efficiency for Sustainable Crop Production: A Focus on Wheat. *AIMS Microbiol.* **2017**, *3*, 413. [[CrossRef](#)]
3. Kumar, A.; Verma, H.; Singh, V.K.; Singh, P.P.; Singh, S.K.; Ansari, W.A.; Yadav, A.; Singh, P.; Pandey, K. Role of *Pseudomonas* sp. in Sustainable Agriculture and Disease Management. In *Agriculturally Important Microbes for Sustainable Agriculture*; Meena, V.S., Mishra, P.K., Bisht, J.K., Pattanayak, A., Eds.; Springer: Singapore, 2017; pp. 195–215.
4. Bender, S.F.; Wagg, C.; van der Heijden, M.G. An Underground Revolution: Biodiversity and Soil Ecological Engineering for Agricultural Sustainability. *Trends Ecol. Evol.* **2016**, *31*, 440–452. [[CrossRef](#)] [[PubMed](#)]
5. Vessey, J.K. Plant Growth Promoting Rhizobacteria as Biofertilizers. *Plant Soil* **2003**, *255*, 571–586. [[CrossRef](#)]
6. Kumar, A.; Patel, J.S.; Meena, V.S. Rhizospheric Microbes for Sustainable Agriculture: An Overview. In *Role of Rhizospheric Microbes in Soil*; Meena, V., Ed.; Springer Nature: Singapore, 2018; pp. 1–31.
7. Patel, T.S.; Minocheherhomji, F.P. Plant Growth Promoting Rhizobacteria: Blessing to Agriculture. *Int. J. Pure Appl. Biosci.* **2018**, *6*, 481–492. [[CrossRef](#)]
8. Bashan, Y.; de-Bashan, L.E.; Prabhu, S.; Hernandez, J.-P. Advances in Plant Growth-Promoting Bacterial Inoculant Technology: Formulations and Practical Perspectives (1998–2013) (A Marshner Review). *Plant Soil* **2014**, *378*, 1–33. [[CrossRef](#)]

9. Tabassum, B.; Khan, A.; Tariq, M.; Ramzan, M.; Khan, M.S.I.; Shahid, N.; Aaliya, K. Bottlenecks in Commercialisation and Future Prospects of PGPR. *Appl. Soil Ecol.* **2017**, *121*, 102–117. [[CrossRef](#)]
10. Shaikh, S.; Sayyed, R. Role of Plant Growth-Promoting Rhizobacteria and their Formulation in Biocontrol of Plant Diseases. In *Plant Microbes Symbiosis: Applied Facets*; Arora, N.K., Ed.; Springer: New Delhi, India, 2015; pp. 337–351.
11. Sohaib, M.; Zahir, Z.A.; Khan, M.Y.; Ans, M.; Asghar, H.N.; Yasin, S.; Al-Barakah, F.N. Comparative Evaluation of Different Carrier-Based Multi-Strain Bacterial Formulations to Mitigate the Salt Stress in Wheat. *Saudi J. Biol. Sci.* **2020**, *27*, 777–787. [[CrossRef](#)]
12. Sudjana, B.; Jingga, A.; Simarmata, T. Enriched Rice Husk Biochar Ameliorant to Increase Crop Productivity on Typic Hapludults. *Glob. Adv. Res. J. Agric. Sci.* **2017**, *6*, 108–113.
13. Seema, K.; Mehta, K.; Singh, N. Studies on the Effect of Plant Growth Promoting Rhizobacteria (PGPR) on Growth, Physiological Parameters, Yield and Fruit Quality of Strawberry Cv. Chandler. *J. Pharmacogn. Phytochem.* **2018**, *7*, 383–387.
14. Elbeltagy, A.; Nishioka, K.; Suzuki, H.; Sato, T.; Sato, Y.-I.; Morisaki, H.; Mitsui, H.; Minamisawa, K. Isolation and Characterization of Endophytic Bacteria from Wild and Traditionally Cultivated Rice Varieties. *Soil Sci. Plant Nutr.* **2000**, *46*, 617–629. [[CrossRef](#)]
15. Aravind, R.; Kumar, A.; Eapen, S.; Ramana, K. Endophytic Bacterial Flora in Root and Stem Tissues of Black Pepper (*Piper Nigrum* L.) Genotype: Isolation, Identification and Evaluation against Phytophthora Capsici. *Lett. Appl. Microbiol.* **2009**, *48*, 58–64. [[CrossRef](#)]
16. Aloo, B.N.; Mbega, E.R.; Makumba, B.A.; Hertel, R.; Daniel, R. Molecular Identification and in Vitro Plant Growth-Promoting Activities of Culturable Potato (*Solanum Tuberosum* L.) Rhizobacteria in Tanzania. *Potato Res.* **2021**, *64*, 67–95. [[CrossRef](#)]
17. Arora, N.; Kumar, V.; Maheshwari, D. Constraints, development and future of the inoculants with special reference to rhizobial inoculants. In *Innovative Approaches in Microbiology*; Maheshwari, D.K., Dubey, R.C., Eds.; Singh and Singh: Dehradun, India, 2001; pp. 241–245.
18. Joshi, M.; Setty, P.T.K. *A Text Book of Irrigation and Water Management*; Kalyani Publishers: Ludhiana, India, 2005.
19. Walkley, A.; Black, I.A. An Examination of the Degtjareff Method for Determining Soil Organic Matter, and a Proposed Modification of the Chromic Acid Titration Method. *Soil Sci.* **1934**, *37*, 29–38. [[CrossRef](#)]
20. Bremner, J.M.; Mulvaney, C.S. Nitrogen-total. In *Methods of Soil Analysis*; Agronomy Monographs; American Society of Agronomy: Madison, WI, USA, 2015; Volume 5, pp. 595–624.
21. Tran, S.; Simard, R. Mehlich III–Extractable Elements. In *Soil Sampling and Methods of Analysis*; Carter, M.R., Ed.; CRC Press: Boca Raton, FL, USA, 1993; pp. 43–49.
22. Jackson, M. *Soil Chemical Analysis*, 2nd ed.; Prentice Hall of India: New Delhi, India, 1973.
23. Chaurasia, S.; Gupta, A.D.; Gupta, D. *Handbook of Water, Air and Soil Analysis*; International Science Congress Association: Chhattisgarh, India, 2014.
24. Abd El-Fattah, D.A.; Eweda, W.E.; Zayed, M.S.; Hassanein, M.K. Effect of Carrier Materials, Sterilization Method, and Storage Temperature on Survival and Biological Activities of Azotobacter Chroococcum Inoculant. *Ann. Agric. Sci.* **2013**, *58*, 111–118. [[CrossRef](#)]
25. Vishwakarma, K.; Kumar, V.; Tripathi, D.K.; Sharma, S. Characterization of Rhizobacterial Isolates from Brassica Juncea for Multitrait Plant Growth Promotion and Their Viability Studies on Carriers. *Environ. Sustain.* **2018**, *1*, 253–265. [[CrossRef](#)]
26. Gade, R.M.; Chaithanya, B.H.; Khurade, K.C. A Comparative Study of Different Carriers for Shelflife of Pseudomonas Fluorescens. *Bioscan* **2014**, *9*, 287–290.
27. Arora, N.K.; Ekta, K.; Narain, R.; Maheshwari, D.K. Sawdust as a Superior for Production of Multipurpose Bioinoculant Using Plant Growth Promoting Rhizobium Spp and Pseudomonas Spp Strains and Their Impact on Productivity of Trifolium Repense. *Curr. Sci.* **2008**, *95*, 90–94.
28. Shahzad, S.; Khan, M.Y.; Zahir, Z.A.; Asghar, H.N.; Chaudhary, U.K. Comparative Effectiveness of Different Carriers to Improve the Efficacy of Bacterial Consortium for Enhancing Wheat Production under Salt Affected Field Conditions. *Pak. J. Bot.* **2017**, *49*, 1523–1530.
29. Sahu, P.K.; Brahmaaprakash, G.P. Formulations of Biofertilizers—Approaches and Advances. In *Microbial Inoculants in Sustainable Agricultural Productivity: Functional Applications*; Singh, D.P., Singh, H.B., Prabha, R., Eds.; Springer: New Delhi, India, 2016; Volume 2, pp. 179–198. ISBN 978-81-322-2644-4.
30. Khavazi, K.; Rejali, F.; Seguin, P.; Miransari, M. Effects of Carrier, Sterilization Method, and Incubation on Survival of Bradyrhizobium Japonicum in Soybean (*Glycine Max* L.) Inoculants. *Enzyme Microb. Tech.* **2007**, *41*, 780–784. [[CrossRef](#)]
31. Mahdi, S.S.; Hassan, G.; Samsoun, S.; Rather, H.; Dar, S.A.; Zehra, B. Biofertilizers in Organic Agriculture. *J. Phytol.* **2010**, *2*, 42–54.
32. Rebah, B.F.; Prevost, D.; Yezza, A.; Tyagi, R.D. Agro-Industrial Waste Material and Wastewater Sludge for Rhizobial Inoculant Production: A Review. *Bioresour. Technol.* **2007**, *98*, 3535–3546.
33. Zayed, M.S. Advances in Formulation Development Technologies. In *Microbial Inoculants in Sustainable Agricultural Productivity, Functional Applications*; Singh, D.P., Singh, H.B., Prabha, R., Eds.; Springer: New Delhi, India, 2016; pp. 219–237.
34. Melin, P.; Håkansson, S.; Eberhard, T.H.; Schnürer, J. Survival of the Biocontrol Yeast Pichia Anomala after Long-Term Storage in Liquid Formulations at Different Temperatures, Assessed by Flow Cytometry. *J. Appl. Microbiol.* **2006**, *100*, 264–271. [[CrossRef](#)]
35. Pandey, P.; Maheshwari, D.K. Bioformulation of Burkholderia Sp. MSSP with a Multispecies Consortium for Growth Promotion of Cajanus Cajan. *Can. J. Microbiol.* **2007**, *53*, 213–222. [[CrossRef](#)] [[PubMed](#)]

36. Bahadur, I.; Maurya, B.; Roy, P.; Kumar, A. Potassium-Solubilizing Bacteria (KSB): A Microbial Tool for K-Solubility, Cycling, and Availability to Plants. In *Plant Growth Promoting Rhizobacteria for Agricultural Sustainability*; Kumar, A., Meena, V., Eds.; Springer: Singapore, 2019; pp. 257–265.
37. Chandrasekaran, B.; Annadurai, K.; Somasundaram, E. *A Text Book of Agronomy*; New Age International Publishers: New Delhi, India, 2010.
38. Itelima, J.U.; Bhang, W.J.; Onyimba, I.A. A Review: Biofertilizer; a Key Player in Enhancing Soil Fertility and Crop Productivity. *J. Microbiol. Biotechnol. Rep.* **2018**, *2*, 22–28.
39. Lobo, C.B.; Juárez Tomás, M.S.; Viruel, E.; Ferrero, M.A.; Lucca, M.E. Development of Low-Cost Formulations of Plant Growth-Promoting Bacteria to Be Used as Inoculants in Beneficial Agricultural Technologies. *Microb. Res.* **2019**, *219*, 12–25. [[CrossRef](#)] [[PubMed](#)]
40. Malusá, E.; Sas-Paszt, L.; Ciesielska, J. Technologies for Beneficial Microorganisms Inocula Used as Biofertilizers. *Sci. World J.* **2012**, *2012*, 491206. [[CrossRef](#)]
41. Phiromtam, M.; Mala, T.; Srinives, P. Effect of Various Carriers and Storage Temperatures on Survival of *Azotobacter Vinelandii* NDD-CK-1 in Powder Inoculant. *Mod. Appl. Sci.* **2013**, *7*, 81–89.
42. Sandikar, B.M.; Awasthi, R.S. Preparation and Shelf-Life Study of *Pseudomonas* and *Bacillus* Bioformulations against Phytopathogenic *Pythium* and *Fusarium* Species. *Int. J. Plant Prot.* **2010**, *2*, 251–254.
43. Thirumal, G.; Reddy, R.S.; Triveni, S.; Nagaraju, Y.; Prasannakumar, B. Screening of Native Rhizobia and *Pseudomonas* Strains for Plant Growth Promoting Activities. *Int. J. Curr. Microbiol. App. Sci.* **2017**, *6*, 616–625. [[CrossRef](#)]
44. Nakkreen, S.; Fernando, D.W.G.; Siddiqui, Z.A. Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases. In *Biocontrol and Biofertilization*; Siddiqui, Z.A., Ed.; Springer: Dordrecht, Netherlands, 2005; pp. 257–296.
45. Chakraborty, A.; Hu, C.X. Lending Relationships in Line-of-Credit and Nonline-of-Credit Loans: Evidence from Collateral Use in Small Business. *J. Financ. Intermediation* **2006**, *15*, 86–107. [[CrossRef](#)]
46. Vassilev, N.; Vassilev, M.; Lopez, A.; Martos, V.; Reyes, A.; Maksimovic, I.; EichlerLöbermann, B.; Malusá, E. Unexploited Potential of Some Biotechnological Techniques for Biofertilizer Production and Formulation. *Appl. Microb. Biotechnol.* **2015**, *99*, 4983–4996. [[CrossRef](#)]
47. Arora, N.K.; Tiwari, S.; Singh, R. Comparative Study of Different Carriers Inoculated with Nodule Forming and Free Living Plant Growth Promoting Bacteria Suitable for Sustainable Agriculture. *J. Pharm. Chem. Biol. Sci.* **2014**, *2*, 143–149.
48. Du Toit, P.J.; Olivier, S.P.; van Biljon, P.L. Sugar Cane Bagasse as a Possible Source of Fermentable Carbohydrates. Characterization of Bagasse with Regard to Monosaccharide, Hemicellulose, and Amino Acid Composition. *Biotech. Bioeng.* **1984**, *26*, 1071–1079. [[CrossRef](#)] [[PubMed](#)]
49. Arora, N.K.; Maheshwari, D.K.; Khare, E. Plant growth promoting rhizobacteria: Constraints in bioformulation, commercialization and future strategies. In *Bacteria and Plant Health*; Maheshwari, D.K., Ed.; Springer: Berlin, Germany, 2010; pp. 97–116.
50. Bharti, N.; Sharma, S.K.; Saini, S.; Verma, A.; Nimonkar, V.; Prakash, O. Microbial Plant Probiotics: Problems in Application and Formulation. In *Probiotics and Plant Health*; Kumar, V., Kumar, M., Sharma, S., Prasad, R., Eds.; Springer: Singapore, 2017; pp. 317–335.
51. Ma, Y. Seed Coating with Beneficial Microorganisms for Precision Agriculture. *Biotechnol. Adv.* **2019**, *2019*, 107423. [[CrossRef](#)]
52. Kumar, V. Characterization, Bio-Formulation Development and Shelf-Life Studies of Locally Isolated Bio-Fertilizer Strains. *Oct. J. Environ. Res.* **2014**, *2*, 32–37.