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Proceeding paper

Effect of Different Carriers and Storage Temperatures on the Viability of *Bacillus thuringiensis* B9 and *Bacillus pacificus* B11 Isolated from Tomato (*Solanum lycopersicum* L.) Rhizosphere

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Abstract: The present study aimed to evaluate the use of agricultural waste as carriers in the bioformulation of two plant growth promoting bacteria (PGPR), *Bacillus thuringiensis* B9 and *Bacillus pacificus* B11 isolated from the tomato rhizosphere, and to determine the effect of different carriers and storage temperatures on bacterial survival. Three carriers, namely palm nut shell biochar, coffee pulp and soil, were inoculated with one of the strains, dehydrated and stored at ambient and refrigeration temperatures (4 °C). Bacterial survival was evaluated for 150 days at 30-day intervals. The results showed that the number of bacterial cells present in the bioformulations decreased progressively with storage time at room temperature, but at refrigeration temperature the bacterial population initially decreased before increasing until reaching its maximum population at 90 days and gradually decreasing afterward. Although the coffee pulp and biochar carriers stored at 4 °C retained the viability of the bacterial strains as well as possible, the formulations stored at room temperature also remained viable

Keywords: Bioformulation; carriers; coffee pulp; biochar; soil; storage temperature; PGPB

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1. Introduction

In recent years, to increase agricultural production, farmers have been forced to make greater use of chemical fertilizers and pesticides. However, intensive and repeated application of these inputs has contributed to degrading the environment and soil fertility [1] and increasing greenhouse gas emissions [2]. For sustainable agriculture, it is important to reduce dependence on these chemical products, which have harmful effects on soil microbial life, particularly the bacteria present in the rhizosphere of crops [3]. Some of these bacteria are capable of improving plant growth through various biochemical mechanisms such as phosphate solubilization, phytohormone production and nitrogen fixation [3]. A large population of these bacteria would help reduce the use of chemical input. Researchers have developed formulations using appropriate carriers. The viability of bacterial cells is strongly influenced by the material used as a carrier and storage temperature. Therefore, this study aims to evaluate the use of agricultural waste (coffee and oil palm) as a carrier and storage temperature on the viability of *Bacillus pacificus* and *Bacillus thuringiensis*.

2. Materials and Methods

2.1. Bacterial Strains

Two bacterial strains were used, Bacillus thuringiensis B9 and Bacillus pacificus B11. These strains were chosen based on their efficacy in improving tomato plant growth of tomato plants in a previous study [4].

2.2. Preparation of Carriers

Three types of solid carriers, namely, soil, biochar, and coffee pulp, were used to evaluate their potential uses for the bioformulation of PGPB. The different carriers were crushed, sieved using a 300 mesh sieve, and then dried to a constant mass at 70 °C in an oven. The different carriers were autoclaved for two consecutive days at 121 °C for 20 min.

2.3. Determination of the Physical and Chemical Properties of Carriers

The physicochemical properties of the carriers, such as organic matter, exchangeable bases, cation exchange capacity, and assimilable phosphorus present in the carriers, were analyzed using the standard methods recommended by Pauwels [5]. Moisture content and water holding capacity were determined on the basis of dry mass using the protocol described by Mwangi [6]. The acidity content (pH) of the carriers was measured using the ratio 1:2.5 (w/v).

2.4. Preparation of Bioformulations

Strains B9 and B11 were reactivated in nutrient agar medium and incubated at 37 °C for 48 h. A cell suspension adjusted to 10^{11} CFU/mL was used for the preparation of bioformulation using the modified protocol of Tripti [7]. A 30 mL cell suspension of each strain was mixed with 200 g of sterilized carrier contained in polyethylene bags. After drying the formulations at 40 °C in an oven to a constant mass, the survival of the strain was assessed using the nutrient agar dilution-suspension method described by Rapilly [8]. The bags were closed, leaving an empty space of around 35% to allow aeration of the bacteria. The bags were divided into two groups and incubated in the dark, one at 4 °C and the other at room temperature. Each treatment was repeated three times in a completely randomized design.

2.5. Assessment of Bioformulation Viability as A Function of Time

The shelf life of the bioformulations was evaluated at 30-day intervals for 150 days. Similarly, the moisture content and pH of the carriers were measured during the storage period. The number of viable cells present in each bioformulation was estimated using the dilution-suspension method as described by Rapilly [8]. Briefly, one gram of each bioformulation was introduced into 9 mL of sterile distilled water and vigorously vortexed to remove bacterial cells from the carrier. Decimal dilutions were then made up to dilution 10^{-7} and 100 µL of dilutions 10^{-5} and 10^{-7} were spread on nutrient agar with three repetitions for each of these dilutions. Petri dishes containing the inoculated medium were incubated at 37 °C for 48 h. The number of colonies was counted, and the number of CFU g^{-1} of bioformulation was determined using the formula proposed by Somasegaran [9]. Number of CFU g-1 = Number of colonies × dilution factor × volume of inoculum

2.5. Statistical Analysis of the Data

The number of CFUs per g of the different carriers was logarithmically transformed to improve the homogeneity of the variance. When the data followed the normal distribution, the analysis of variance and the statistical difference between the means were performed using Duncan's multiple test at the 5% probability threshold.

3. Results

3.1. Physicochemical Characteristics of Carriers

The physicochemical properties of the carriers varied from one to another (Table 1).The carriers were slightly acidic with a pH of 5.6 and 6.0, respectively, for the soil and

coffee pulp, but basic (pH = 8.1) for the biochar. The water holding capacity (WHC) varied from 62% (soil) to 101.43% (coffee pulp).

Table 1. Physicochemical characteristics of the carriers.

Characteristics	Biochar	Coffee pulp	Soil
Physical characteristics			
WHC	92.57 ± 8.31	184.32 ± 14.71	63.13 ± 16.09
Moisture content after drying (%)	15.20 ± 0.13	14.87 ± 0.91	15.41 ± 0.55
Particle size (%)			
<106 µm	14.86	30.17	20.80
106-216 µm	13.43	24.28	15.47
216-500 µm	40.76	31.49	40.43
500 µm -1 mm	30.95	10.80	23.30
>1 mm	0	3.26	0
Chemical characteristics			
Ph	8.1 ± 0.0	6 ± 0.0	5.6 ± 0.0
N (%)	0.21	1.68	0.01659
P	159.03 mg/kg	0.05 (%)	35.65 mg/kg
K	1.76 meq/100g	1.15 (%)	4.25 mg/kg
OM (%)	81	95.1	4.97
OC (%)	40.5	47.55	2.88

WHC: water holding capacity; OM: organic matter; OC: organic carbon.

After inoculation and drying at 40 °C, the water content of the carriers varied between 14 and 15%. During storage, the water content varied according to the carrier, temperature, and storage time (Figure 1). In general, the water content of the carriers decreased with storage time, with the exception of the pulp carrier stored at refrigeration temperature, where an increase in water content was observed with storage time.

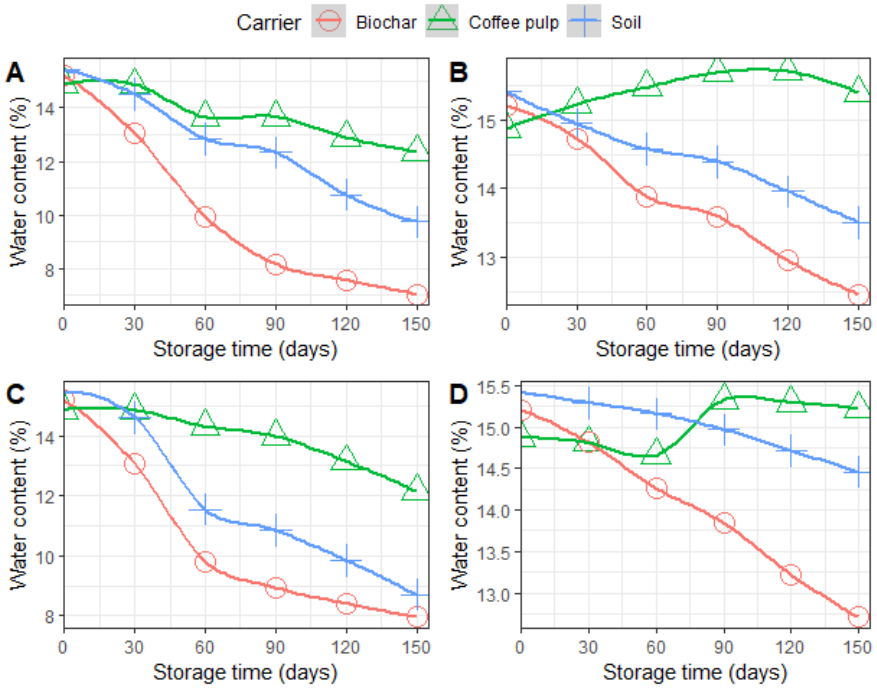

Figure 1. The water content of the carriers as a function of the storage time of the Bacillus thuringiensis B9 strains at ambient (A) and refrigerated (B) temperatures; and Bacillus pacificus B11 at ambient (C) and refrigerated (D) temperatures.

3.2. Survival Rate of Strains After Drying

After inoculation of the carriers and drying in an oven at 40 °C, the survival rate of the bacterial inocula was assessed. It ranged from 22.3 to 89.1% depending on the carrier and strain (Table 2).

Table 2. Strain survival rate after carrier drying.

Carrier	**Coffee pulp**		Biochar		Soil	
Strain	**B9**	**B11**	B9	B11	B9	B11
Survival rate (%)	89.1	73.4	79.4	63.1	22.3	39.8

3.3. Viability of Deformations as a Function of Carriers and Storage Temperature

The viability of the strains *B. thuringiensis* B9 and *B. pacificus* B11 on different carriers (coffee pulp, biochar, and soil) and storage temperatures (ambient and refrigerated) was studied for 150 days and expressed as log CFU per gram of carrier (Figure 2). It varied according to the carrier used, the temperature, and the storage time. The coffee pulp carrier and biochar showed the best survival rates for the bacterial inocula after 150 days of storage.

At room temperature, the number of bacterial CFU present in the bioformulations decreased progressively with storage time of up to 150 days (Figures 2A and C). A more significant decrease in strain viability was observed in some carriers than in others. At refrigerated temperatures, the bacterial population first decreased, then gradually increased until it reached its maximum population at 90 days, before gradually decreasing to 150 days (Figures 2B and D).

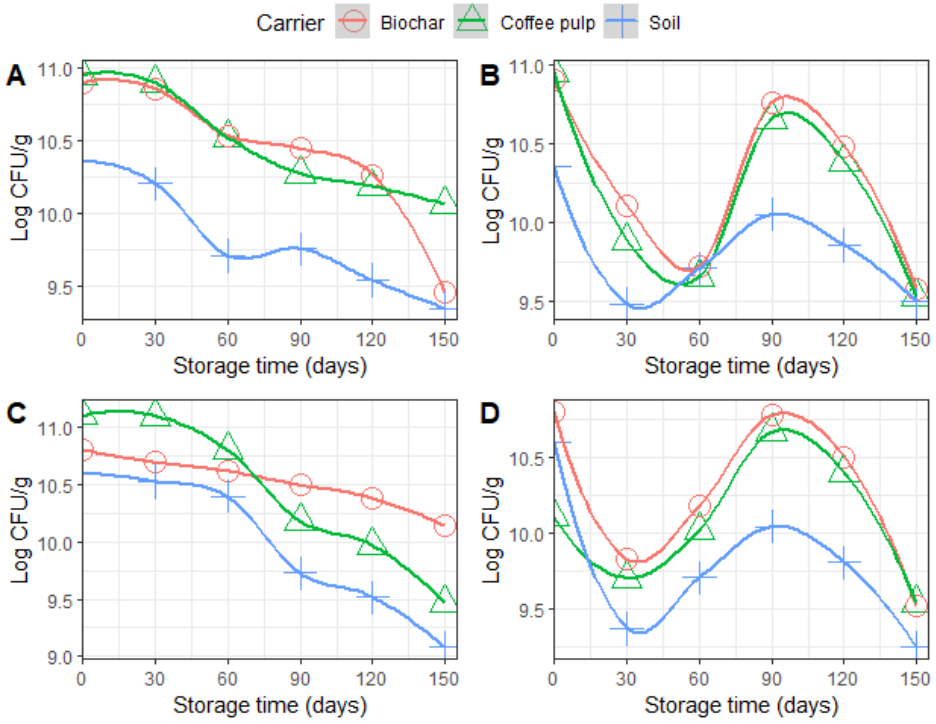

Figure 2. Viability of bioformulations at different storage intervals of *Bacillus thuringiensis* B9 strains under ambient (A) and refrigerated (B) temperatures and *Bacillus pacificus* B11 under ambient (C) and refrigerated (D) temperatures.

4. Discussion

4.1. Effect of the Type of Carrier on the Survival of Bacterial Inocula

The highest survival rates were obtained with coffee pulp, while the lowest were obtained with soil. The high viability rate of the strains after dehydration could be explained by the fact that strains of the genus Bacillus are generally resistant to a certain temperature. At a certain temperature, they are capable of producing endospores or capsules that are highly resistant to heat or environmental stress [10].

4.2. Effect of Carrier Type on Bacterial Inocula Survival

The viability of bacterial inocula is influenced by several factors, including the type of carrier material, storage temperature, and storage time. In general, a significant decrease in the number of viable cells was observed in the bioformulations during storage time. This difference in capacity between carriers can be attributed to the difference in their physicochemical properties [11,12]. The coffee pulp-based bioformulation showed the highest bacterial survival rate, followed by biochar and soil after 150 days. This may be due to the fact that coffee parchment exhibited the highest water holding capacity, high organic matter content, near-neutral pH, low C/N ratio, and low water content loss during storage, which provided a favorable microenvironment for bacteria during the storage period. These characteristics reflect the properties of good carriers [13].

4.3. Effect of Storage Temperature on Bacterial Inocula Survival

In the present study, the carriers were stored at room temperature and at 4 °C. The carriers stored at refrigeration temperature showed the highest cell viability. This difference in results could be explained by the fact that cold storage reduced the rate of loss of water from carriers. A significant loss of water from the carriers would have accelerated the dehydration of the bacterial cells, causing them to die over time [14]. Furthermore, storage under refrigerated conditions would have reduced the metabolic and physiological activities of the bacteria while maintaining a level of humidity that would have kept the bacteria alive for a little longer [15].

5. Conclusion

In conclusion, the storage time and viability of the bacterial inocula were influenced by the type of carrier and the storage temperature. Although the pulp carrier and biochar stored at 4 °C retained the viability of the bacterial strains to the greatest extent possible, the formulations stored at room temperature remained viable, enabling them to be used in agricultural production systems that do not have a costly cold chain.

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