LONGEVITY OF *PSEUDOMONAS* STRAINS IN PEAT AND TALC BASED BIOFORMULATIONS

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ABSTRACT

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The study was conducted in Plant Protection Department at University Putra Malaysia (UPM), Malaysia in 2014 to formulate *Pseudomonas* bacterial strains UMB20 and BMB42 for long time preservation. Two carrier materials viz. peat and talc were used in the formulation and stored under room and 4°C. Efficacy of the carriers under storage condition in sustaining the viability of the bacterial strains was assessed monthly. Antagonistic ability of the strains was evaluated at the end of storage period. After 6 months of storage, peat formulation was found to retain the viable cells of 1.05×10^8 cfug^{-1} for strain mixture which was followed by 0.86×10^8 and $0.82\times10^8$for individual BMB42 and UMB20, respectively. Talc retained the viability of bacteria up to 3 months with 0.008×10^8cfu g^{-1}in the strain mixture (UMB20+BMB42) which was followed by 0.003×10^8cfu g^{-1} and 0.001×10^8cfu g^{-1} in BMB42 and UMB20, respectively. Both individuals and strain mixtures were found to exhibit the potential of antagonism (59.26-61.11%) against *Rhizoctonia solani* as like their fresh cultures. This study suggested that indigenous *Pseudomonas* bacteria isolated from plants can be formulated in peat and talc and preserved in peat under 4°C for up to 6 months.

Key words: *Pseudomonas* bacterial strains, peat and talc.

INTRODUCTION

Bacterial species under the genus of *Pseudomonas* like *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa* and *P. aureofaciens* have drawn worldwide attention for their potency to suppress different diseases of plants (Karthikeyan *et al*. 2006, Suryadi *et al*. 2011) including sheath blight of rice (Mew and Rosales 1986, Gnanamanickam *et al*. 1992). In the above cases, fresh cell cultures grown on media of these bacterial antagonists were used. Although the fresh cell cultures are useful for research purpose but not convenient for commercial use by farmers. However, for long term storage and easy application, bacterial inoculum should be immobilized in suitable carriers. Reports on the development and optimization of different bioformulations adding appropriate carrier materials (Krishnamurthy and Gnanamanickam 1998, Ali *et al*. 2001) and adhesives (Schmidt *et al*. 2001) are available. Different organic and inorganic materials are being used as carriers to develop formulation in the form of either powder, solid or liquid (Chakravarty and Kalita 2011) and the suitable carriers should be applicable as powder and granular form (Motsara *et al*. 1995, Somasegaran and Hoben 1994). Peat and talc are the most common and extensively used carrier materials for formulation of bacterial antagonists (Mathivanan *et al*. 2005, Gnanamanickam 2009)

Viability of inoculum in a formulation for a certain length of time is important thing to be considered for commercialization of the technology (Bashan 1998). Peat has been reported as effective carrier to retain the viability of bacterial antagonists. For examples, peat based formulation of *Azospirillum brasilense* had a shelf life up to 4 months with the population of 10^7cfug^{-1} (Bashan 1998). Vidhyasekaran and Muthamilan (1995) reported that the shelf life of *P. fluorescens* in peat-based formulation was maintained up to 8 months with 2.8×10^6cfug^{-1}. Shelf life of *P. chlororaphis* (PA23) and *B. subtilis* (CBE4) in peat carriers was retained for more than six months (Kavitha *et al*. 2003, Nakkeeran *et al*. 2004). Several published reports on talc based formulations of *Pseudomonas* bacteria are available (Suryadi *et al*. 2013, Gupta and Dohroo 2014). Kloepper and Schroth (1981) reported that fluorescent pseudomonads remained viable for two months of storage in talc mixture with 20% xanthum gum at 4°C. Isolate of *P.*

fluorescens (Pf1) survived up to 240 days in storage (Vidhyasekaran and Muthamilan 1995). Amendment of sucrose (0.72M) in King's B medium increased the population and shelf life of *P. fluorescens* (P7NF, TL3) in talc-based formulation up to 12 months (Caesar and Burr 1991). *Pseudomonas putida* strain 30 and 180 survived up to 6 months in talc based formulations (Bora *et al*. 2004).

The objectives of the present study were1) to formulate the *Pseudomonas* bacterial strains using peat and talc 2) to see the effect of carriers on the viability of two *Pseudomonas* bacterial strains under different conditions and 3) to determine the antagonistic activity against *Rhizoctonia solani*.

MATERIALS AND METHODS

Bacterial inoculants

Two promising *Pseudomonas* bacterial strains *P. fluorescens* (UMB20) and *P. asplenii* (BMB42) were obtained from our previous study (Akter *et al.* 2014). Bacterial isolates were isolated from rice plant samples collected from different rice growing areas of Malaysia. Preliminary selection of the bacteria was done using dual culture assay(Gupta *et al.* 2001).One loop of 48 hour old culture of bacterial isolates was streaked at 1 cm from the outer side of 9-cm PDA petri plates. Three day-old *Rhizoctonia solani* culture was placed at the centre of PDA petri plates 2.5 cm apart from the bacteria. The plates were incubated for 3 days at 28±2°C and the growth of fungus was measured in the presence or absence of any bacterial isolates.For secondary selection extra cellular metabolites assay was done according to the method described by Intana *et al.* (2008) with little modifications. Bacterial isolates were grown on Nutrient broth and incubated at 28±2°C on a rotary shaker (150 rpm) for 4 days. After centrifuge the supernatant was filtrated with germ filter (d=0.22µm). Culture filtrate from each isolate was mixed with PDA medium at the rate of 25% (v/v) and poured on petri plates. Three day-old culture of *R. solani* plug was placed directly to the solid medium. After 3 days of incubation at 28±2°C, diameter of fungal colony was measured compared with the control.Peat was purchased from local agriculture shop in Malaysia and the talc was obtained from the Plant Protection Department, University Putra Malaysia, Malaysia.

Compatibility test of the bacterial strains

For mixture formulation with the two strains, compatibility of UMB20 and BMB42 with each other was tested on nutrient agar medium according to Muthukumar *et al*. (2010) with slight modification. Both strains were mixed with the medium at 2ml/18ml^{-1} (v/v) before pouring into the petri plates. After solidifying the media, sterile filter paper discs (13 mm) were placed on the solid medium at 2 places maintaining a distance of 1.5 and 2 cm away from the periphery of the plates. For UMB20, 10 µl of BMB42 (OD_{600} = 0.12) was dropped on the first disc and for the comparison 10 µl of sterile water was dropped on to the second disc. For BMB42, the same procedure as above was followed. The plates were incubated at room temperature (28±2ºC) for 72 hours and presence or absence of inhibition zones around the discs was observed. Three replications were maintained for each strain and the test was repeated once.

Preparation of bacterial cell suspension

Inoculants were prepared according to Husen *et al.* (2011) with modifications. A loop full of each isolate from nutrient agar (NA) medium was transferred to conical flask containing 100 ml of nutrient broth (NB) and incubated in a water bath shaker at 130 rpm for 48 hour at 30°C. After 48 hour of incubation the concentrations of bacterial cells in the suspensions were adjusted to an absorbance of 0.5 which was equivalent to 1×10^9 cfuml^{-1} with the aid of UV-visual spectrophotometer at 600nm. For preparation of mixture, bacterial isolates were grown separately at the same condition and equal volume of each strain were mixed together and adjusted to 1×10^9as in the case of individual strains.

Preparation of formulation

Peat and talc were amended with $CaCO_3$ at the rate of 4% (w/w) to increase the pH level of the substrate carriers and carboxymethyl cellulose (1:10: w/w) was used as an adhesive. All the components were mixed together in an auto clavable plastic bag and sterilized at 121°C for 60 minutes at 15 psi. The mixtures were then spread with the help of a sterile spatula onto sterile plastic sheets under aseptic conditions and allowed to dry. Then 48 hour old bacterial suspensions (1×10^9cfu ml^{-1}) grown in NB were added to the carrier materials at the rate of 40% (v/w) and mixed thoroughly. Powder and granule formulations were made of talc and peat, respectively. Then the peat based mixture was passed through the sieve (2.8 mm diameter) to form pellet shaped formulation. Talc was spread over sterile aluminum foil to dry and retained 20% moisture content in a drying oven at 30°C. The formulations were divided into two sets, with one set was kept at 4°C and other one kept at room temperature (28±2ºC).

Enumeration of viable cell of bacteria in formulation under storage condition

Viable cells of bacteria in the formulations were enumerated at one month intervals for up to 6 months using the method described by Abdel-Kader *et al.*

(2012). Bacterial colonies were counted following the plate count technique. Suspensions were prepared by adding one gram of each formulated bacteria to 250 ml volume flasks containing 99 ml of sterile water. In the case of pellet (peat) formulation, the flasks were shaken for 1 hour at 130 rpm using a bench shaker. After settling down, 10^{-1} to 10^{-4} serial dilutions were made from the suspensions. A volume of 0.1 ml from the 10^{-2} and 10^{-3} dilutions was spread on to 9-cm petri plates containing 15 ml of NA medium with the help of a sterile angled glass rod. After 24-48 hour of incubation bacterial colonies were counted and colony forming units (cfu) per gram formulation was calculated using the following formula(Chakravarty and Kalita 2011):

$$\text{cfu/g} = \frac{\text{Total number of cfu}}{\text{Volume plated} \times \text{Dilution factor} \times \text{g/mL}}$$

Evaluation of antagonistic ability of formulated stored bacterial strains against *Rhizoctonia solani*

To determine the antagonistic ability of the formulated bacteria against *Rhizoctonia solani*, 50 µl of the individual strains and strain mixture suspensions were streaked on to the PDA plates using a micropipette 2 cm away from the fungal plug (Nandakumar *et al.* 2001). A four millimeter diameter disc from a 3 day old culture of *Rhizoctonia solani* was placed at the center of the plate. After 72 hours of incubation the radial growth of the pathogen was measured. Plates streaked with sterile water served as control. The growth of the pathogen in the presence or absence of bacteria was measured and expressed as percent growth inhibition.

Experimental design and statistical analysis

Data obtained from the experiments were subjected to analysis of variance (ANOVA) with SAS software version 9.2(SAS, Institute Cary, NC, 1987) by analysis of variance and differences among the treatments were determined using least significant difference (P ≤0.05).

RESULTS AND DISCUSSION

Compatibility of bacterial isolates

In strain mixture formulation compatibility is the important thing. *Pseudomonas* bacterial strains of UMB20 and BMB42 were tested for their compatibility *in vitro*. No inhibition zone was found around the disk in the medium (Figure 1). This result suggested that these bacterial strains were compatible to each other.

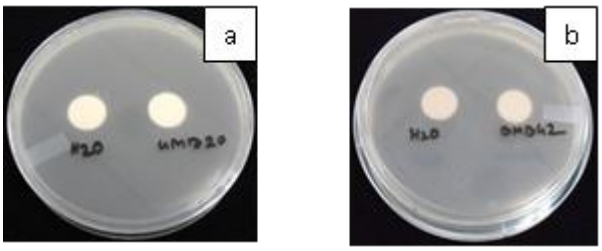

Figure 1: Compatibility of UMB20 and BMB42 with each other: (a) filter paper disc inoculated with UMB20 on the nutrient agar amended with BMB42 and (b) filter paper disc inoculated with BMB42 on nutrient agar amended with UMB20.

Effect of storage period on the viability of bacterial strains in carrier materials

The average results obtained from the viability test of bacterial strains in peat based formulation under the two different storage conditions are presented in Table 1 and Table 2. Initially, the average populations of UMB20, strain mixture (UMB20+BMB42) and BMB42 were recorded as in peat formulations ranged from cfu g^{-1} 2.92 × 10^8, cfu g^{-1}2.90 × 10^8 andcfu g^{-1} 2.80 × 10^8 respectively.

In the first month of storage in peat at 4°C, significantly high cfu g^{-1} (2.72 × 10^8) was recovered from the strain mixture formulation (Table 1). The cfu g^{-1} was found to be almost the same for UMB20 and BMB42 (1.50 × 10^8 and 1.49 × 10^8cfu g^{-1}, respectively). Cell counts after 2, 3, 4 and 5 months of storage revealed that the population of both individuals and strain mixture formulation declined slowly following the same trend. After 6 months of storage significantly high number of colonies (1.05×10^8cfu g^{-1}) was recorded in the strain mixture formulation, while 0.82 ×10^8 and 0.86 ×10^8cfu g^{-1} were recorded with UMB20 and BMB42, respectively (Table 1).

Table 1: Viability of bacterial inoculants in peat formulation at 4°C storage condition

Bacterial isolates	Bacterial colony number count per gram of dry carrier material (×10^8)						
	Storage period (month)						
	0	1	2	3	4	5	6
UMB20	2.92a	2.50b	1.86c	1.65c	1.34c	1.05c	0.82b
BMB42	2.80b	2.49b	2.06b	1.80b	1.49b	1.21b	0.86b
UMB20+ BMB42	2.91a	2.72a	2.20a	1.88a	1.62a	1.31a	1.05a
LSD	0.10	0.09	0.12	0.06	0.08	0.06	0.10

Mean of four replications. Mean values within columns followed by the same letter are not significantly different (P ≤0.05).

Viability test results under the room temperature storage conditions for the peat formulation (Table 2) revealed that the number of viable cells declined rapidly within two months in both individual as well as the strain mixture formulation. In the first month, significantly high number of viable cells (1.88×10^8cfu g^{-1}) was recovered from the BMB42 formulation, while the same number of viable cells (1.27×10^8cfu g^{-1}) was recovered from the UMB20 and strain mixture formulations. In the second month, significantly high number of cfu g^{-1} (0.72×10^8) was recorded in UMB20 which was followed by 0.58×10^8 (BMB42) and 0.50×10^8 (UMB20+BMB42). After 3 months of storage the number of viable cells of all strains (single or combined) showed little decrease and at the end of 4 months, a similar trend with decreasing number of viable cells was observed for the single strains and the strain mixture. After 6 months of storage both single strains and strain mixture formulations had negligible number of cfu g^{-1} and there were no significant differences in the number of viable cells among them.

Table 2: Viability of bacterial inoculants in peat formulation at room temperature storage condition

Bacterial isolates	**Bacterial colony number count per gram of dry carrier material ($\times10^8$)**						
	Storage period (month)						
	0	1	2	3	4	5	6
UMB20	2.92a	1.27b	0.72a	0.50b	0.23b	0.018b	0.0015a
BMB42	2.80b	1.88a	0.58b	0.61a	0.41a	0.015b	0.0016a
UMB20+ BMB42	2.91a	1.27b	0.50c	0.34c	0.25b	0.023a	0.0017a
LSD	0.10	2.57	0.04	0.04	0.04	0.094	0.003

Mean of four replications. Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).

Monthly viability test results revealed that viable colonies of bacterial strains either single or combinations gradually declined by prolonging the storage under both conditions in peat. Comparatively the decreasing rate of viable cells was slightly slower at 4°C storage condition while they were stored under room temperature condition in the same materials. Lashmipriya and Sivakumar (2013) stated that low temperature condition especially at 4-10°C may retarded the multiplication and metabolic activities of bacteria resulted in reduced consumption of nutrient and reduced moisture content of carrier which favoured the retention of viable cells. Declining of viable cells under room temperature (25-28°C) might be happened due to drying process and toxin production in this temperature (Cigdem and Merih 2005).Our results are the agreement with the following reports. Peat based formulation of *Azospirillum brasiliense* had a shelf life upto4 months and this population was sufficient for successful plant inoculation (Garcia and Samiento 2000) and the shelf life of *P. fluorescens* up to 8 months (Vidhyasekaran and Muthamilan 1995). Shelf life *B. subtilis* (CBE4) in peat carriers was retained for more than six months (Kavitha *et al.* 2003, Nakkeeran *et al.* 2004). Khabbaz and Abbasi (2014) reported that the two *Pseudomonas* and one *Bacillus subtilis* bacteria survived of in peat and talc formulations decreased over time at room temperature, but the populations remained above the 10^8 cfu g^{-1} during the 180-day storage period.

The effects of talc based powder formulations on viability of bacteria (cfu g^{-1}) under the two storage conditions are presented in Table 3 and Table 4. After one month of storage at 4°C, the highest cfu g^{-1} (1.04×10^8) was recorded with UMB20, which was followed by 0.60×10^8 and 0.57×10^8 which were obtained with UMB20+BMB42 and BMB42, respectively. After 2 months, the average cell counts were very low compared to initial numbers and were statistically similar for the bacterial strains singly or as mixture. After 3 months, the highest number of cfug^{-1}(0.008×10^8) was recorded in the strain mixture (UMB20+BMB42) formulation which was followed by 0.003×10^8cfu g^{-1} and 0.001×10^8cfu g^{-1} in BMB42 and UMB20, respectively (Table 3).

Table 3: Viability of bacterial inoculants in talc formulation at 4°C storage condition

Bacterial isolates	**Bacterial colony number count per gram of dry carrier material ($\times10^8$)**						
	Storage period (month)						
	0	1	2	3	4	5	6
UMB20	2.04c	1.04a	0.03a	0.0015c	-	-	-
BMB42	2.20b	0.57b	0.05a	0.003b	-	-	-
UMB20+BMB42	2.31a	0.60b	0.04a	0.008a	-	-	-
LSD	0.06	0.08	0.01	0.007			

Mean of four replications. Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$).-= No viable cell of bacteria.

In case of room temperature storage of the talc formulation, a similar trend with declining number of viable cells of bacteria was observed (Table 4). After one month, the recovered cfu g^{-1} for the strain mixture was statistically the highest (1.13×10^8), and this was followed by UMB20 (0.94×10^8) and BMB42 (0.77×10^8). After 3 months, the recovered cfu g^{-1} for strain mixture was still statistically the highest (0.05×10^8), while the lowest was observed with BMB42 (0.01×10^8) which was statistically similar to UMB20 (0.02×10^8cfu g^{-1}).

Table 4: Viability of bacterial inoculants in talc formulation at room temperature storage condition

Bacterial isolates	**Bacterial colony number count per gram of dry carrier material ($\times 10^8$)**						
	Storage period (month)						
	0	1	2	3	4	5	6
UMB20	2.04c	0.94b	0.07b	0.02b	-	-	-
BMB42	2.20b	0.77c	0.07b	0.01b	-	-	-
UMB20+BMB42	2.31a	1.13a	0.19a	0.05a	-	-	
LSD	0.06	0.05	0.02	0.01			

Mean of four replications. Mean values within columns followed by the same letter are not significantly different (P ≤0.05).-= No viable cell of bacteria.

Talc has supported the viability of bacterial strains up to 3 months at both 4°C and room temperature condition of storage. Our results are disagreement with Salaheddin *et al.* (2010), they reported that *P. fluorescens* isolates Pf32 and Pf93 and *Bacillus subtilis* isolate B49 survived in the talc-based formulation for more than 90 days. Fluorescent pseudomonads remained viable for two months of storage in talc mixture with 20% xanthum gum at 4°C (Kloepper and Schroth 1981). In our formulation, we amended CMC with talc as sticker and bacterial isolates remained viable for 3 months at 4°C and room temperature condition. Our results did not corroborate with the results of Bora *et al.* (2004). According to them *P. putida* strain 30 and 180 survived up to 6 months in talc based formulations.

The data on percent viability losses in bacterial strains that occurred during the six months period in the peat formulation are presented in figure 2. The losses ranged from 6.61-71.82% in peat based formulations at 4°C (Figure 2A) and 32.78-99.94% at room temperature (Figure 2B) storage conditions. In the first month of storage, the percent viability losses for all individual strains and strain mixture at room temperature were recorded around 3 folds higher than at the 4°C storage conditions. Percent viability losses in formulated bacterial strains increased with prolonged storage period. At the end of storage period percent viability losses of individual and strain mixture were ranging from 63.68-71.82% at 4°C storage conditions (Figure 2A). In the room temperature storage conditions, they were found to lose their viability by almost 100%.

In case of talc based formulations, percent viability losses of bacterial strains were found to be higher in both refrigerated (Figure 2C) and room temperature (Figure 2D) storage conditions. It was observed that, the rate of loss in viability was rapid under both storage conditions. After one month of storage both individuals and combined strain loss in their viability by 50% and in the 3rd month the loss in viability was around 100% under both storage conditions.

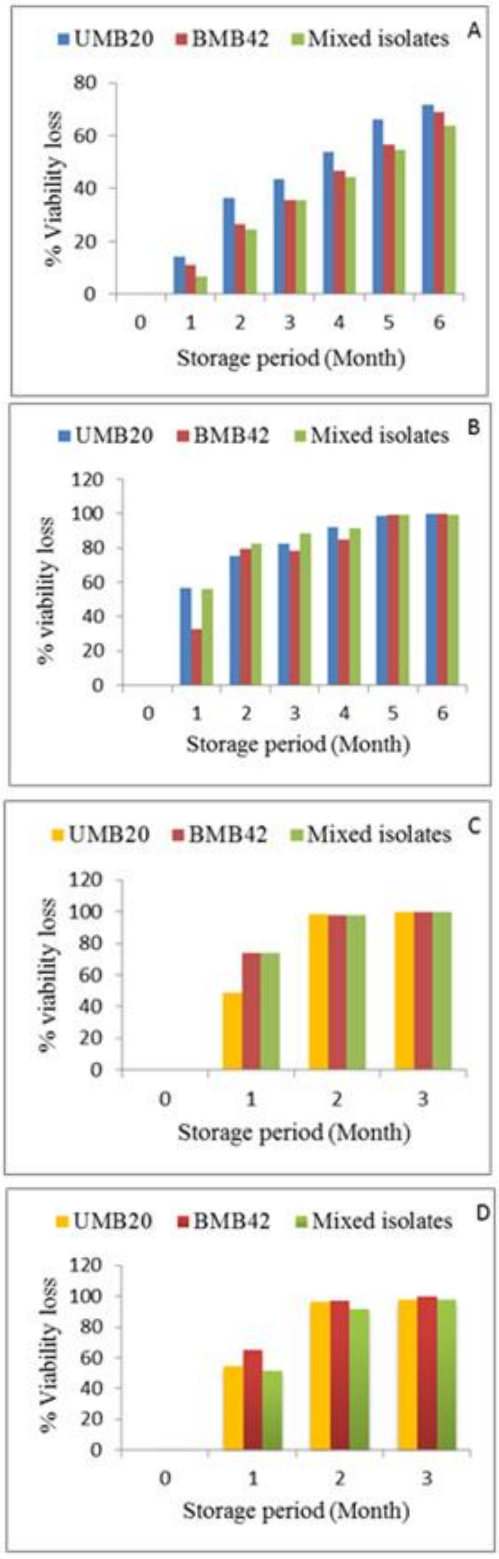

Figure 2. Percent viability losses of formulated bacterial strains as individuals and strain mixture in peat and talc at 4°C and room temperature (28±2°C) conditions: (A) in peat at 4°C; (B) in peat at room temperature (28±2°C)condition; (C) in talc at 4°C and (D) in talc at room temperature (28±2°C)condition.

Antagonistic activities of bacterial strains

Presented data in Table 5 showed the antagonistic ability of bacterial strains stored for 6 months (in peat) and 3 months (in talc) in different carrier materials against sheath blight pathogen *Rhizoctonia solani* compared to the fresh cultures of the same strains. The overall percent growth reduction of the pathogen obtained from the antagonism test of the formulated strains ranged from 59.26-61.11%. Data revealed that no significant differences were observed in the antagonistic activities of the individual and combinations of strains stored either at 4°C or room conditions in both the carrier materials. At the end of storage period of respective formulations, negligible numbers of viable cells were found with bacterial strains in both formulations. It is interesting to note that no significant differences were found in antagonistic ability of formulated bacterial strains compared with the fresh cultures of them. These activities are correlated with their efficacy in the management of plant diseases (Radjacommare *et al.*2007). From these results it can be inferred that the antagonistic ability of bacterial antagonist is not depended on the number of population but on active viability.

Table 5. Effect of storage period on antagonistic activity of individual and mixed bacterial isolates in peat and talc formulation against the growth of *Rhizoctonia solani* under 4°C and room temperature (RT)condition

Carrier and storage condition	**Bacterial isolates (after 6 months in peat and 3 months of storage in talc carriers)**	**Growth reduction (%) of *Rhizoctiniasolani* over control**
Peat + 4°C	UMB20	60.74a
	BMB42	60.75a
	UMB20+BMB42	61.11a
Peat + RT	UMB20	59.26a
	BMB42	59.62a
	UMB20+BMB42	60.37a
Talc + 4°C	UMB20	60.00a
	BMB42	59.26a
	UMB20+BMB42	60.74a
Talc +RT	UMB20	60.74a
	BMB42	59.26a
	UMB20+BMB42	60.37a
Fresh cultures	UMB20	61.11a
	BMB42	60.37a
	UMB20+BMB42	60.37a
Control		0
LSD		2.95
CV (%)		4.26

Mean of four replications. Mean values within columns followed by the same letter are not significantly different ($P \leq 0.05$). RT= Room temperature; Fresh culture= 48 hours old culture of bacterial strain grown on nutrient agar; Control=No bacterial culture with fungal culture on Potato Dextrose agar plate.

CONCLUSION

Peat formulation retained significantly high number of colonies (1.05×10^8cfu g^{-1}) of strain mixture (UMB20 +BMB42), while 0.82×10^8 and 0.86×10^8cfu g^{-1} were with UMB20 and BMB42, respectively for 6 months at 4°C. In talc formulation they remain viable until 3 months. Therefore, bacterial isolates can be preserved in peat at 4°C. The formulation should be tested further for their efficacy in plant-soil association under glass house and field condition.

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