STUDY ON VIABILITY OF FUNGAL BIOAGENTS IN VACUUM PACKING COMPARED WITH CONVENTIONAL PACKING IN DIFFERENT STORAGE CONDITIONS

Abstract

The shelf life and viability of the bioagents are influenced by several factors, including the type of nutrients in the formulation, moisture content and the ability of propagules to withstand adverse climatic conditions. There are numerous commercial formulations of *Trichoderma* species primarily used to combat soil-borne diseases. The present study was conducted to understand the optimal storage conditions suitable for these bioagents. By this study, where the viability in vacuum packing and conventional packing were compared, it was observed that the vacuum packing did not offer any advantages over conventional packing methods. Additionally, storing the bioagents in a refrigerator at 4 °C was more effective compared to other storage conditions, such as room temperature (27 ± 2 °C), incubator (24 ± 2 °C) and deep freezer (-20 °C).

Keywords: *Viability*, *Trichoderma* sp., *Vacuum packing, Conventional packing*

Introduction

'Biological control' can be broadly defined as the reduction of the harmful effects caused by certain organisms, commonly known as natural destroyers. In the context of plant pathology, however, it specifically refers to the intentional use of introduced or native biotic organisms aside from disease resistant host plants to decrease the activities and populations of one or more plant pathogens (Pal and Gardener, 2006).

Synthetic pesticides contribute to environmental pollution, while excessive chemical use can diminish soil fertility and increase erosion. Biological control can be viewed as a multifaceted approach, with its success depending on the rhizosphere effectiveness of microbial inoculants (Naik *et al.,* 2016) their interactions with the native microbiota, their ability to compete for nutrients, and their adaptability to changing environmental conditions, all of which enhance the host plant's protection against pathogens (Pal and Gardener, 2006). Biological control of plant diseases involves the suppression of plant pathogen populations by living organisms (Heimpel and Mill, 2017). While synthetic pesticides are the fastest way to control

plant diseases, they can be highly detrimental to both human health and the environment. These chemicals directly impact flora and fauna, disrupting beneficial microorganisms essential for plant. Therefore, there is a need to explore alternative to chemical pesticides for managing plant diseases. Biological control serves as a viable alternative method for disease management that supports an eco-friendly environment (Rajkumar *et al.,* 2018b). It plays a crucial role in managing plant diseases without harming beneficial organisms and also enhances soil fertility.

Fungi are systematically and biologically diverse, with many species capable of inhibiting the growth of pathogenic fungi that are detrimental to plant development (Bheemaraya *et al*., 2013). Significant attention has been given to developing fungal strains as biocontrol agents for plant diseases, particularly those from the genus *Trichoderma*. Other fungi, such as *Penicillium*, *Gliocladium*, *Aspergillus* and *Saccharomyces*, (Tariq *et al*., 2020) have also shown antagonistic effects against various plant fungal pathogens, including *Alternaria*, *Pythium*, *Aspergillus*, *Fusarium*, *Rhizoctonia*, *Phytophthora*, *Botrytis*, *Pyricularia* and *Gaeumannomyces* (Rajkumar *et al.,* 2018a). *Trichoderma* sp. is a significant biocontrol agent effective against a wide range of aerial and soil-borne plant pathogens (Divya *et al.,* 2015), making it a potential biopesticide for field and greenhouse applications. Understanding the optimal storage conditions for these bioagents is essential to maintain a healthy number of colony forming units. Hence, the present study was undertaken to assess the viability of *Trichoderma* sp. bioagents in vacuum packing compared with conventional packing methods.

Materials and methods

Mass production of the fungal bioagents *i.e., Trichoderma harzianum, T. asperellum and T. asperelloides* was done using talc powder as a carrier material. Fungal bioagents were initially grown on Potato Dextrose Broth (PDB) medium and then transferred to talc powder. For this purpose, flasks (500 ml capacity) containing 250 ml PDB was inoculated with 5 mm disc of fungal bioagents separately to the respective sets of flasks. The flasks were incubated at 28 ± 1 ºC for 15 days. After fifteen days of incubation, well grown culture of the fungal bioagent was mixed thoroughly with the talc powder. Visual observations on fungal growth were made daily and mixed thoroughly by shaking (Singh *et al.,* 2015).

The formulations after being prepared was packed and sealed under two different packing, *i.e.*, Normal and vacuum packing. Conventional packing and vacuum packing were carried out in Biocontrol Unit and Vacuum Packaging Laboratory, University of Agricultural Sciences, Raichur respectively. These were later subjected to four different storage conditions *i.e.*, normal room temperature (27 ± 2 ºC), incubator (24 ± 2 ºC), refrigerator (4 ºC) and deep freezer (-20 ºC). The factorial completely randomized design (FCRD) was employed to perform a statistical analysis of the collected data. The viability of all these was checked at every fifteen days interval for six months.

Results and discussion

Studies on viability of *Trichoderma harzianum* under vacuum packing compared with conventional packing

The results revealed the effect of various storage temperatures and packaging on the viability and shelf life of *Trichoderma harzianum*, which has been presented in Table 1. It was observed that the number of colonies gradually decreased from 15 days to 180 days after incubation under all the storage conditions (plate 1).

At 180 days after incubation, under normal packing, the number of colonies per gram was significantly found to be more under refrigerator condition (4 ºC) (20×10^6) followed by deep freezer (-20 ºC) (15×10^6). The least number of colonies was found under room temperature (27 ± 2 ºC) (12.67×10^6).

Number of colonies in vacuum packing after 180 days of storage was found be significantly higher in refrigerator condition (4 ºC) (16.67×10^6). Whereas, all other conditions were on par with each other showing 10×10^6 cfu/g.

In a comparative analysis of packing methods under varying temperature and duration, it was noted that a significantly higher quantity of fungal colonies were observed in samples subjected to refrigerator condition (4 ºC) and normal packing at 180 days (20×10^6), followed by samples stored in refrigerator condition (4 ºC) in vacuum packing (16.67×10^6) (Fig. 1).

Rai and Tewari (2016) observed in their study that lower temperatures could increase the shelf life of *Trichoderma harzianum* bioproducts. Where, at room temperature (15-35^{0}C) there was a gradual decline in the cfu of *Trichoderma* in all the formulations up to six months and

thereafter sudden decline was observed. The formulations of *Trichoderma* stored at 4^0C revealed that at refrigerator (4^0C) there was a gradual decline in the cfu of *Trichoderma* in all the formulations up to eleven months and thereafter sudden decline was observed. Daryaei *et al*. (2016) also suggested that *T. atroviridae* stored at lower temperatures had a longer shelf life compared to those kept at higher temperatures suggesting that this decrease in viability might be linked to increased metabolic activity at elevated temperatures. Consequently, a higher metabolic rate could negatively impact the shelf life of the biocontrol agent.

Studies on viability of *Trichoderma asperellum* under vacuum packing compared with conventional packing

The results revealed the effect of various storage temperatures and packaging on the viability and shelf life of *Trichoderma asperellum,* which has been presented in Table 2 and plate 2. It was observed that the number of colonies gradually decreased from 15 days to 180 days after incubation under all the storage conditions.

After 180 days it was it was observed that under normal packing, the number of colonies per gram was significantly more in refrigerator condition (4 ºC) (20×10^6) followed by room temperature (27 ± 2 ºC) (16.67×10^6). The least number of colonies of 15×10^6 was recorded under incubator condition (24 ± 2 ºC).

Under vacuum packing, the number of colonies per gram was found to be significantly higher in refrigerator condition (4 ºC) (16.67×10^6) followed by room temperature (27 ± 2 ºC) (13.33×10^6) and deep freezer (-20 ºC) (13.33×10^6) which were on par with each other. The least number of colonies were observed under incubator condition (24 ± 2 ºC) (12×10^6).

When considering both the normal packing and vacuum packing at different temperatures and days of interval, at 180 days after storage significantly more colonies were observed under refrigerator condition (4 ºC) at normal packing (20×10^6), followed by room temperature and normal packing (16.67×10^6) which was on par with those stored under refrigerator condition (4 ºC) under vacuum packing (16.67×10^6). The least number of colonies of 12×10^6 was observed at the incubator conditions (24 ± 2 ºC) under vacuum packing (Fig. 2).

Viability of *Trichoderma* sp. is known to be greatly affected by temperature conditions. This was evident from the differences in viable cfu observed at two different temperatures of storage by Manandhar *et al.* (2018). The products kept at ambient temperature conditions were

found to lose their viability rapidly and viable cfu could only be recorded for a maximum of six months. The products stored in refrigerator showed higher viability percentages over the time frame and satisfactory level of cfu was observed for until six months. However, after this period of storage, the viability reduced significantly and by the end of one year, only a maximum of 7.23 per cent of viability was achieved.

Studies on viability of *Trichoderma asperelloides* under vacuum packing compared with conventional packing

The results revealed the effect of various storage temperatures and packaging on the viability and shelf life of *Trichoderma asperelloides*, which has been presented in Table 3 and plate 3. It was observed that the number of colonies gradually decreased from 15 days to 180 days after incubation under all the storage conditions.

Under normal packing, the number of colonies was found to be 20×10^6 at 180 days under refrigerator condition recording significantly the highest cfu/g followed by 16.67×10^6 cfu/g under room temperature (27 ± 2 ºC). The least number of colonies of 11.67×10^6 was noticed in the samples stored in deep freezer for the duration of 180 days.

Under vacuum packing, the greater number of colonies of 15×10^6 was observed after 180 days of incubation in refrigerator condition (4 ºC) followed by those stored in incubator (24 ± 2 ºC) (10×10^6). The least cfu/g of 8.33×10^6 was observed under room temperature (27 ± 2 ºC).

When comparing the effects of normal packing and vacuum packing under various temperature conditions and different days of interval, a significant difference in fungal colony count was observed. Specifically, the highest colony count of 20×10^6 was recorded under refrigerator conditions (4 ºC) in normal packing, followed by 16.67×10^6 in room temperature (27 ± 2 ºC) under normal packing. The least cfu/g of 8.33×10^6 was observed under room temperature (27 ± 2 ºC) which was vacuum packed (Fig. 3).

According to a study by Trivedi *et al.* (2005), storing microorganisms at low temperatures helps reduce nutrient depletion, prevents the accumulation of toxic metabolites and minimizes moisture loss, thus supporting long-term storage. However, they found that both conidial and mycelial formulations nearly lost viability when stored at -20 °C after just one month. They suggested that this temperature could harm cell structure due to gradual cooling. At

room temperature, viability was completely lost within six months. The study also indicated that oxygen levels in the experimental packaging could differ between standard and vacuum packaging, which affected the viability of resident commensal microbes. Notably, vacuum packaging did not show any advantages over conventional packaging methods. This may be because vacuum packaging failed to create a completely anaerobic environment, as residual oxygen likely remained in the product. The relatively high permeability of polyethylene, the material used for vacuum packaging, could contribute to this issue compared to other packaging materials (Tripathi *et al.*, 2014; Karimi *et al*., 2011).

Conclusion

When both vacuum packing and conventional packing were compared and the number of cfu/g was assessed it was observed that the vacuum packing didn't provide any advantage over normal or conventional packing. Conventional packing proved to be better and more reliable for storing these bioagents to maintain good number of colonies than vacuum packing.

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Authors' contribution

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Conflict of interest

Authors have declared that there is no conflict of interest.

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Table 1. Viability of *Trichoderma harzianum* under different packaging and storage conditions

No. of days	Normal packing (Cfu/g × 10^6)					Vacuum packing (Cfu/g × 10^6)				
	Room temperature (27 ± 2 °C)	Refrigerator (4 °C)	Incubator (24 ± 2°C)	Deep freezer (-20 °C)	Mean	Room temperature (27 ± 2 °C)	Refrigerator (4 °C)	Incubator (24 ± 2°C)	Deep freezer (-20 °C)	Mean
0 days	65.00	65.00	65.00	65.00	65.00	65.00	65.00	65.00	65.00	65.00
15 days	60.00	58.33	50.00	55.00	55.83	60.00	56.67	50.00	55.00	55.42
30 days	55.00	56.67	45.00	51.67	52.08	51.67	51.67	45.00	50.00	49.58
45 days	50.00	50.00	40.00	48.33	47.08	48.33	46.00	41.00	46.67	45.50
60 days	47.00	47.00	37.67	43.33	43.75	38.33	43.33	40.00	41.67	40.83
75 days	43.33	45.00	35.00	40.00	40.83	33.33	42.67	36.33	40.00	38.08
90 days	41.67	43.67	35.00	37.00	39.33	31.67	42.00	33.00	35.00	35.42
105 days	38.00	42.67	30.00	35.67	36.58	30.00	39.33	30.00	33.33	33.17
120 days	36.67	42.67	27.00	35.00	35.33	26.67	36.00	25.00	31.67	29.83
135 days	30.00	41.33	25.00	30.00	31.58	23.33	35.00	21.33	26.67	26.58
150 days	20.00	40.00	20.00	25.67	26.42	16.33	30.00	16.67	23.33	21.58
165 days	16.67	30.00	19.33	20.00	21.50	12.00	23.33	11.00	15.00	15.33
180 days	12.67	20.00	14.67	15.00	15.58	10.00	16.67	10.00	10.00	11.67
Mean	39.69	44.79	34.13	38.59		34.36	40.59	32.64	36.41	

Factors	S. Em. ±	C.D. at 1 %	Factors	S. Em. ±	C.D. at 1 %
Packaging (P)	0.12	0.44	**P × T**	0.24	0.87
Temperature (T)	0.17	0.62	**P × D**	0.43	1.58
Days (D)	0.30	1.11	**T × D**	0.60	2.22
			P × T × D	0.85	3.14

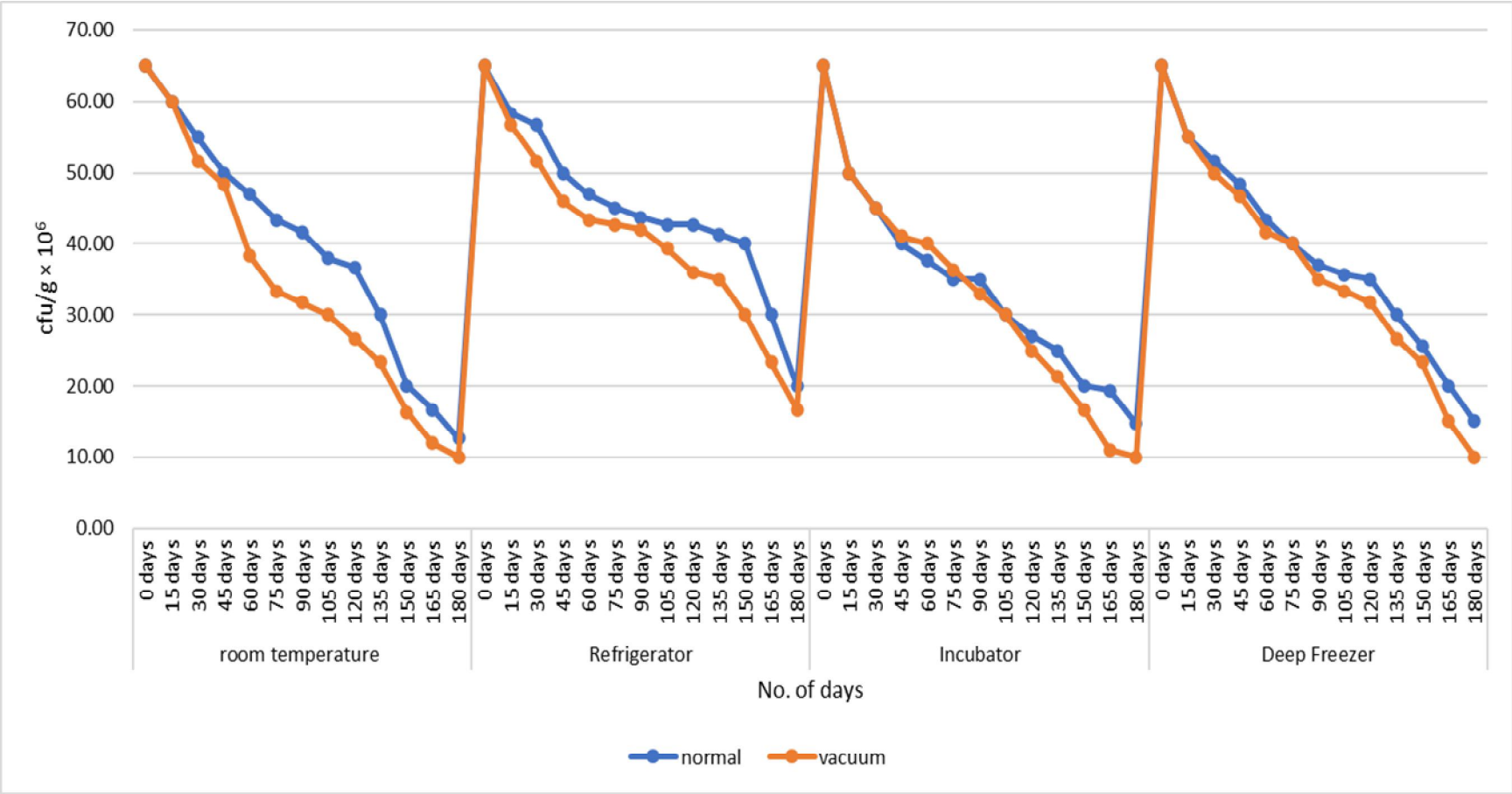
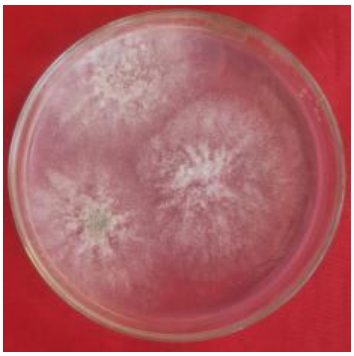
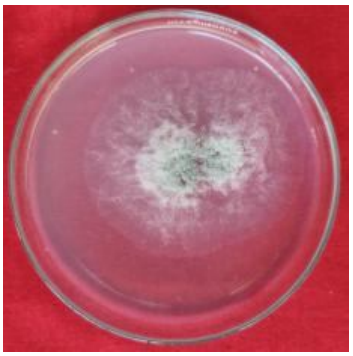


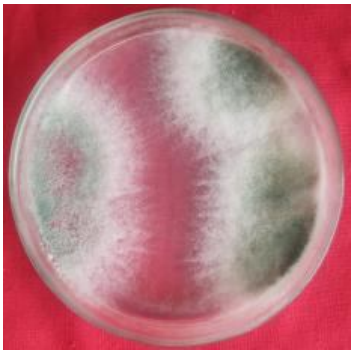
Fig. 1 Viability of *Trichoderma harzianum* under vacuum packing compared with conventional packing



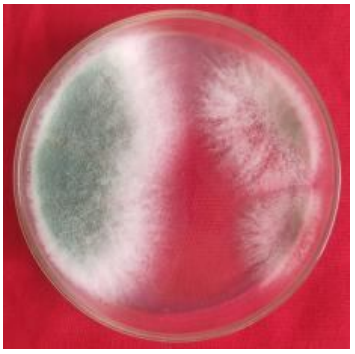
Normal packing(NP)- Room temperature



Vacuum packing (VP) – Room temperature



NP – Refrigerator



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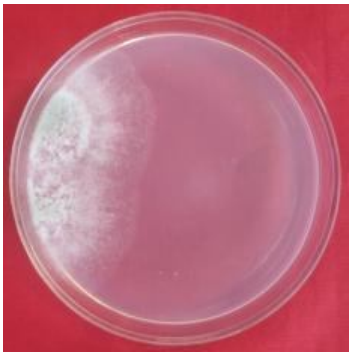
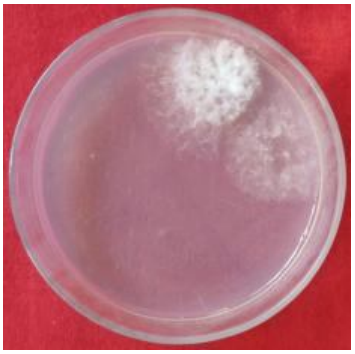


NP – Incubator



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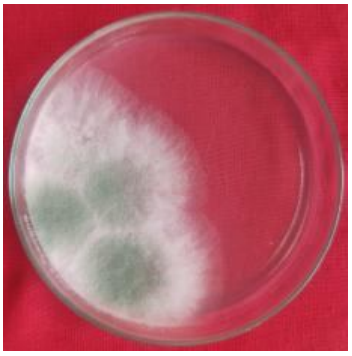
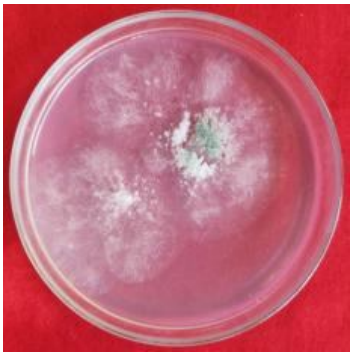


NP - Deep freezer



VP - Deep freezer

Plate 1. Viability of *Trichoderma harzianum* at 120 days at different storage and packing conditions

Table 2. Viability of *Trichoderma asperellum* under different packaging and storage conditions

No. of days	Normal packing (Cfu/g × 10^6)					Vacuum packing (Cfu/g × 10^6)				
	Room temperature (27 ± 2 °C)	Refrigerator (4 °C)	Incubator (24 ± 2°C)	Deep freezer (-20 °C)	Mean	Room temperature (27 ± 2 °C)	Refrigerator (4 °C)	Incubator (24 ± 2°C)	Deep freezer (-20 °C)	Mean
0 days	60.00	60.00	60.00	60.00	60.00	60.00	60.00	60.00	60.00	60.00
15 days	55.00	60.00	45.00	53.33	53.33	50.00	55.00	42.67	50.00	49.42
30 days	48.33	58.67	41.67	50.00	49.67	45.00	51.67	40.00	50.00	46.67
45 days	48.67	55.00	37.67	45.00	46.58	43.33	48.33	33.67	36.67	40.50
60 days	46.33	54.33	30.00	40.00	42.67	40.00	45.00	30.00	35.00	37.50
75 days	45.00	50.00	30.67	30.00	38.92	38.33	43.00	29.00	30.00	35.08
90 days	44.33	45.00	26.67	25.00	35.25	36.33	41.33	25.67	30.00	33.33
105 days	38.33	40.00	26.00	22.67	31.75	35.00	40.00	25.00	25.00	31.25
120 days	40.00	36.67	23.00	22.00	30.42	33.67	34.67	22.67	20.00	27.75
135 days	36.00	30.00	21.00	20.00	26.75	31.00	30.00	21.67	18.33	25.25
150 days	33.00	25.00	20.00	18.00	24.00	29.00	26.00	18.33	16.33	22.42
165 days	26.67	26.67	19.33	17.67	22.58	23.33	20.00	15.00	15.00	18.33
180 days	16.67	20.00	16.00	15.00	16.92	13.33	16.67	12.00	13.33	13.83
Mean	41.41	43.18	30.54	32.21		36.79	39.36	28.90	30.74	

Factors	S. Em. ±	C.D. at 1 %	Factors	S. Em. ±	C.D. at 1 %
Packaging (P)	0.12	0.45	**P × T**	0.23	0.86
Temperature (T)	0.17	0.63	**P × D**	0.44	1.61
Days (D)	0.31	1.14	**T × D**	0.61	2.24
			P × T × D	0.89	3.23

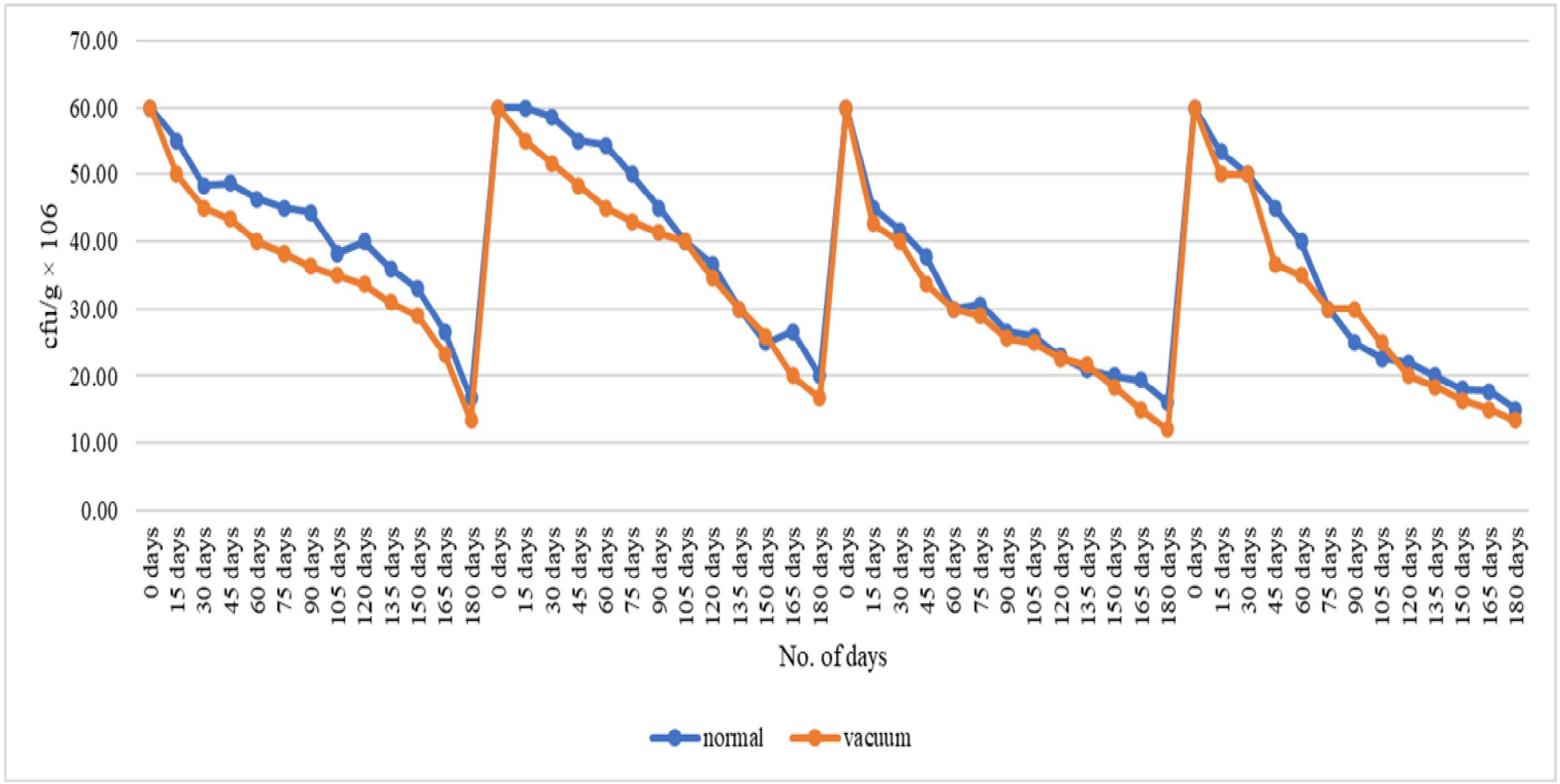
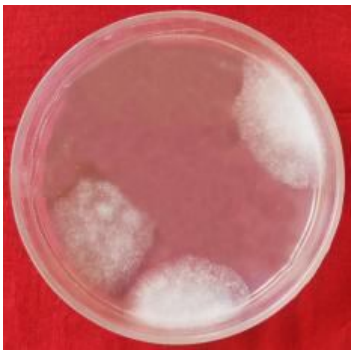
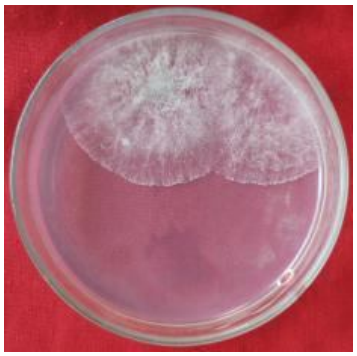


Fig. 2 Viability of *Trichoderma asperellum* under vacuum packing compared with conventional packing



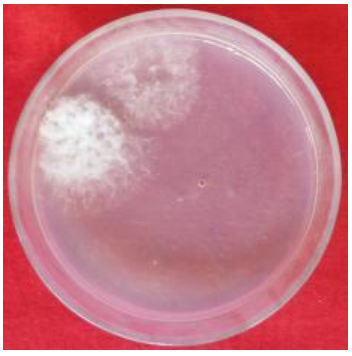
Normal packing(NP)- Room temperature



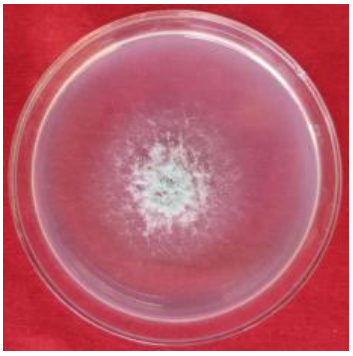
Vacuum packing (VP) – Room temperature

NP – Refrigerator

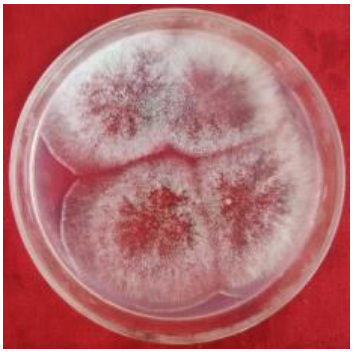
VP – Refrigerator



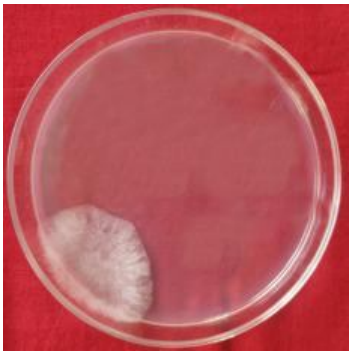
NP – Incubator



VP – Incubator



NP - Deep freezer



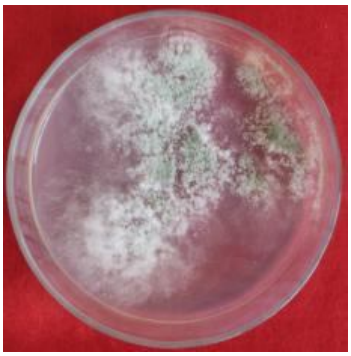
VP - Deep freezer

Plate 2. Viability of *Trichoderma asperellum* at 120 days at different storage and packing conditions

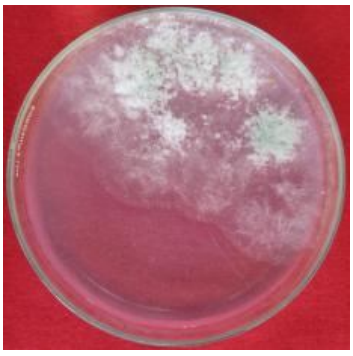
Table 3. Viability of *Trichoderma asperelloides* under different packaging and storage conditions

No. of days	Normal packing (Cfu/g × 10^6)					Vacuum packing (Cfu/g × 10^6)				
	Room temperature (27 ± 2 °C)	Refrigerator (4 °C)	Incubator (24 ± 2°C)	Deep freezer (-20 °C)	Mean	Room temperature (27 ± 2 °C)	Refrigerator (4 °C)	Incubator (24 ± 2°C)	Deep freezer (-20 °C)	Mean
0 days	70.00	70.00	70.00	70.00	70.00	70.00	70.00	70.00	70.00	70.00
15 days	65.00	67.00	60.00	60.00	63.00	67.67	67.67	60.00	66.67	65.50
30 days	63.00	65.00	55.00	56.67	59.92	66.33	66.33	50.00	55.00	59.42
45 days	60.00	60.00	51.67	54.33	56.50	60.00	60.00	46.67	48.33	53.75
60 days	56.33	57.00	50.00	51.00	53.58	55.00	55.00	45.67	53.00	52.17
75 days	50.00	55.00	48.00	54.00	51.75	50.00	53.67	45.00	50.00	49.67
90 days	48.00	53.33	43.33	50.00	48.67	46.00	50.33	40.00	46.00	45.58
105 days	46.33	51.67	40.00	43.33	45.33	40.00	50.33	37.33	40.00	41.92
120 days	40.00	51.00	36.67	45.00	43.17	36.00	45.67	34.33	41.33	39.33
135 days	35.67	48.00	35.00	40.00	39.67	28.33	29.67	30.00	35.00	30.75
150 days	30.00	31.67	30.00	30.00	30.42	20.00	26.67	26.67	25.00	24.58
165 days	20.00	20.67	25.00	23.33	22.25	15.00	17.33	20.00	21.67	18.50
180 days	16.67	20.00	13.33	11.67	15.42	8.33	15.00	10.00	9.33	10.67
Mean	46.23	50.03	42.92	45.33		43.28	46.74	39.67	43.18	

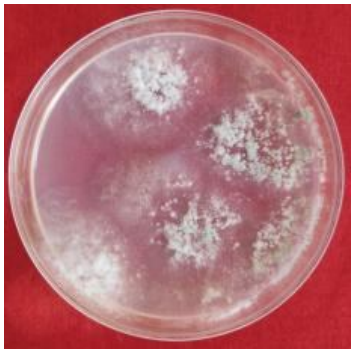
Factors	S. Em. ±	C.D. at 1 %	Factors	S. Em. ±	C.D. at 1 %
Packaging (P)	0.108	0.41	**P × T**	0.216	0.792
Temperature (T)	0.152	0.560	**P × D**	0.39	1.43
Days (D)	0.274	1.010	**T × D**	0.54	2.02
			P × T × D	0.751	2.86



Normal packing(NP)- Room temperature

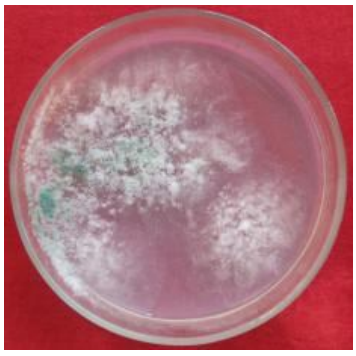


Vacuum packing (VP) – Room temperature

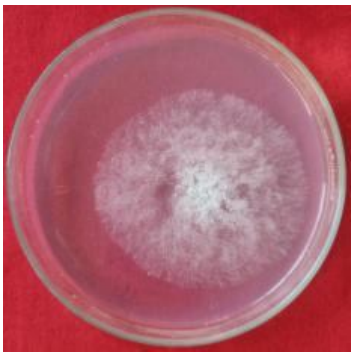


NP – Refrigerator

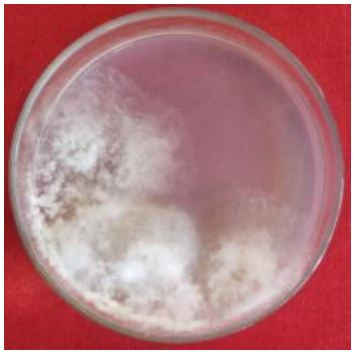
VP – Refrigerator



NP – Incubator



VP – Incubator



NP - Deep freezer

VP - Deep freezer

Plate 3. Viability of *Trichoderma asperelloides* at 120 days at different storage and packing conditions

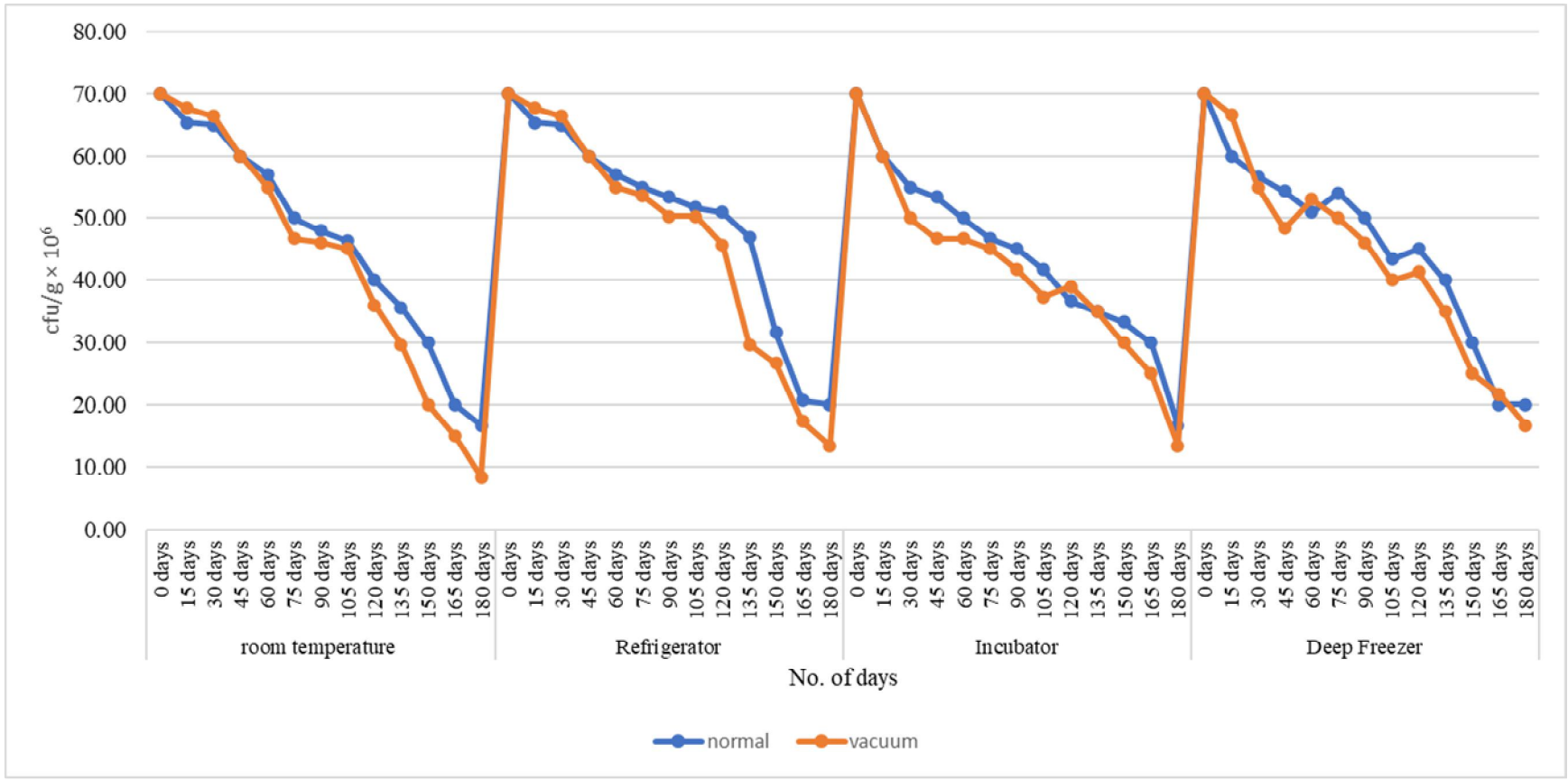


Fig. 3 Viability of *Trichoderma asperalloides* under vacuum packing compared with conventional packing