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A New Carrier based Bioformulation: *Cordyceps militaris* L. (Link)

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ABSTRACT: *Cordyceps militaris* is an entomopathogenic ascomycetous mushroom which is recently gaining attention as a biocontrol agent against plant pests and diseases. But there are no formulations of this biocontrol agent is available in market till date. So, the present study evaluated four different formulations of *C. militaris* using various carrier materials *viz.*, talc, vermiculite-wheat bran, pesta and alginate. The ability of *C. militaris* to sustain over a storage period of 90 days was evaluated by counting the spores at 15 days interval starting from 15 days after inoculation. It was observed that talc and alginate-based formulations performed well throughout the study by sustaining the maximum number of spores even up to 90 days after storage with a spore reduction of 67.70% and 58.33%, respectively at 90 days after storage. Further, the cost of production of each formulation was calculated and talc-based formulation was found to be the best and cost effective as compared to others with an approximate cost of production of Rs.456.00/kg.

Keywords: *Cordyceps militaris*, carrier, talc, vermiculite-wheat bran, pesta, alginate.

INTRODUCTION

Plant pathogens pose a significant threat to global agriculture, leading to reduced crop yields, economic losses, and environmental concerns (Fones *et al.*, 2020; Tripathi *et al.*, 2022; Singh *et al.*, 2023). Traditional methods of plant disease management often involve the use of chemical pesticides, which can have adverse effects on ecosystems and human health. As a sustainable and eco-friendly alternative, the utilization of beneficial microorganisms for biocontrol has gained prominence (Jaiswal *et al.*, 2022). Many fungi and bacteria are being used as potential biocontrol agents against plant pathogens. Although many fungal biocontrol agents are available in market, an emerging biocontrol agent of genus Cordyceps is gaining attention as it can be used as an antagonist against both pests and diseases.

Cordyceps is a genus of insect parasitic fungi that has gained attention not only for its potential medicinal properties but also for its role as a biocontrol agent against biotic stresses caused by insects and pathogens in plants. Cordyceps fungi are known for their ability to infect and kill insects by invading their bodies, altering their behaviour, and eventually producing fruiting bodies that emerge from the host's body (Shang *et al.*, 2015). This same parasitic behaviour is harnessed for biocontrol purposes to target plant pests and diseases.

Cordyceps militaris, a well-known entomopathogenic fungus with various potential health benefits has now gained significant attention towards managing biotic stresses caused by insects and plant pathogens due to the presence of bioactive compounds.

The successful formulation of *C. militaris* is crucial for its commercial application as a biocontrol agent against insects and pathogens. This study aimed to evaluate and determine a suitable carrier-based formulation that maintains the viability and stability of *C. militaris*. Even though many species of Cordyceps is being studied as biocontrol agents against many plant pathogens, none of them were formulated using carrier materials for practical application. The characteristics of an ideal formulation (a) should have more shelf life (b) should be cost effective (c) carrier materials should be easily available and (d) storage facilities should be feasible (Abd El-Fattah *et al.*, 2013).

This study explored a pioneering approach by formulating a carrier material using a combination of talc, vermiculite, wheat bran, pesta grain and sodium alginate to enhance the efficacy of *C. militaris* over a storage period of 3 months (90 days). Further, the cost-effective formulation was also determined by comparing the market cost of carrier materials and also the production cost.

MATERIAL AND METHOD

Collection and maintenance of *C. militaris* culture. *Cordyceps militaris* fungal culture was purchased from Directorate of Mushroom Research (DMR), Solan, Himachal Pradesh and subcultured in potato dextrose agar or Potato Dextrose Broth (PDB) and incubated at 25 ºC.

Formulation of *C. militaris.* Various carrier materials *viz.,* talc, vermiculite-wheat bran, pesta grain and alginate were used for formulating *C. miliaris.* The fungus was grown in PDB and the fermenter biomass was mixed with various carrier materials and stored. The number of spores were observed using Neubauer chamber at 15, 30, 45, 60, 75 and 90 days after preparation of the formulation.

The procedure to prepare the formulations as described by Kumar *et al.* (2014) are given as below:

Talc based formulation. *Cordyceps militaris* grown in PDB was mixed with talc powder @ of 1:2 and 5g of Carboxy Methyl Cellulose (CMC) was added per kilogram of the formulation and mixed well. The mixture was dried to 8 per cent moisture under shade. After drying it was packed in an air tight polythene bag and stored for further study (Photo 1a).

Vermiculite-wheat bran-based formulation. A mixture containing 100 g of vermiculite and 33 g of wheat bran was sterilized in hot air oven at 70 °C for three consecutive days. Then, 20 ml of fermenter was added to it and mixed well and dried in shade. After drying it was packed in an air tight polythene bag and stored for further study (Photo 1b).

Pesta granules-based formulation. Fermenter biomass at the rate of 52 ml was added to 100 g of wheat flour and mixed by gloved hands to form a cohesive dough. The dough was kneaded, pressed and folded by hand several times. Then, one mm thick sheets (pesta) were prepared and air-dried until they broke crisply. After drying, the dough sheet was ground and passed through an eighteen-mesh sieve, and the granules were collected (Photo 1c, Photo 2).

Alginate prills based formulation. Sodium alginate was dissolved in one portion with distilled water (25 g/750 ml), and wheat bran as food base was suspended in another portion (50 g/250 ml). These preparations were autoclaved and when cooled, they were blended together with biomass. The mixture was added dropwise into 1 M $CaCl_2$ solution to form spherical beads (Photo 3) which were washed, air-dried, and added in distilled water and stored at 5 °C (Photo 1 (d)).

Determination of Number of Spores. The total spores were counted in a Neubauer's chamber and the resulting concentrations were expressed as the total number of spores/ml. An aliquot (10 µL) of the diluted formulations (1:10) (w/v) was placed in the Neubauer's chamber by counting of 4 quadrants of each field. The number of spores/ml was calculated for each field and the values were averaged using the following formula given by Aneja (2007).

$$\text{Total no. of spores (per ml)} = \text{No. of spores counted in middle square of grid} \times 10^4$$

Further, the per cent reduction in spore count was calculated by the formula

$$\text{Reduction in spore count (\%)} = \frac{\text{Initial count} - \text{Final count}}{\text{Initial count}} \times 100$$

a. Talc based formulation b. Vermiculite wheat bran based formulation c. Pesta grain-based formulation d. Sodium Alginate based formulation

Photo 1a-d: Various carrier materials prepared for *C. militaris*.

Photo 2: Pesta sheets ground to granules

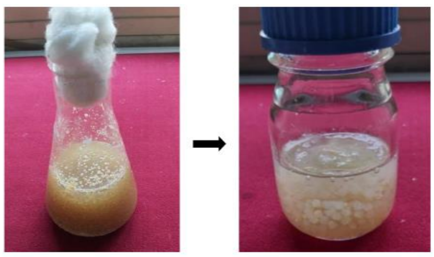

Photo 3: Alginate beads formed using wheat bran and sodium alginate

Cost of Production of Formulations. The cost of production of carrier-based formulations prepared from *C. militaris* was worked out by considering the market cost of the carrier material, labour charge, electricity and packaging materials and the cost effectiveness of each formulation was determined by adding all the cost of individual factors.

Statistical analysis. The experiment was carried out in Completely Randomized design (CRD) with four replications and the data subjected to analysis of variance (ANOVA) and Least Significant Difference (LSD) for testing the treatment significance using statistical package for Social Sciences Version 22.0 (SPSS 22.0) programme. Data were compared using LSD at $p \leq 0.05$.

RESULTS AND DISCUSSION

Spore Count in Different Carrier Materials. Various carrier materials *viz.*, talc- based formulation, vermiculite-wheat bran-based formulation, pesta grain-based formulation and alginate-based formulation were evaluated for their ability to sustain the shelf life of *C. militaris* starting from 15 days up to a storage period of 90 days after inoculation (DAI). The data on spore count is presented in the Table 1. The spore count showed a decreasing trend over the storage period in all the treatments.

At 15 days after inoculation, the highest spore count ($17.03 \pm 0.25 \times 10^6$ spores/g) was observed in talc-based formulation which was significantly higher than rest of the treatments. This was followed by alginate-based formulation with a spore count of $15.30 \pm 0.31 \times 10^6$ spores/g. The next best treatment was vermiculite wheat bran-based formulation with a spore count of $12.23 \pm 0.24 \times 10^6$ spores/g which was statistically at par with pesta grain-based formulation with a spore count of $11.55 \pm 0.12 \times 10^6$ spores/g. Though, the spore count was good at 15 days after inoculation, the count level dropped after this period.

The Fig. 1 depicted the per cent reduction of spore count at various days of estimation from 15 days after inoculation. At 30 and 45 days after inoculation, a similar trend of result was recorded. Among different formulations tested, talc-based formulation was found to be the best at 30 and 45 days after inoculation with a spore count of $15.35 \pm 0.49 \times 10^6$ spores/g and $14.10 \pm 0.44 \times 10^6$ spores/g with a reduction of 9.86 per cent and 17.20 per cent from 15 days, respectively.

The next best treatment was alginate-based formulation with a spore count of $14.13 \pm 0.21 \times 10^6$ spores/g and $12.65 \pm 0.17 \times 10^6$ spores/g respectively at 30 and 45 days after inoculation and there was a reduction of 7.68 per cent and 17.32 per cent from15 days. The least spore count ($9.18 \pm 0.06 \times 10^6$ spores/g) and highest per cent spore reduction (20.56%) was recorded in pesta grain-based formulation at 30 days after inoculation whereas at 45 days the spore count was $8.00 \pm 0.25 \times 10^6$ spores/g and its reduction was 24.98 per cent.

At 60 days after inoculation the least per cent spore reduction (31.54%) was pronounced in alginate-based formulation with a spore count of $10.48 \pm 0.19 \times 10^6$ spores/g. The next best treatment in terms of spore count reduction was talc-based formulation (34.82%) with a spore count of $11.10 \pm 0.32 \times 10^6$ spores/g. However, there was significantly highest spore count at 60 days after inoculation in talc-based formulation. Vermiculite wheat bran-based formulation and pesta grain-based formulation recorded 45.01 per cent and 46.32 per cent of spore reduction with the significantly least spore counts of $6.73 \pm 0.11 \times 10^6$ spores/g and $6.20 \pm 0.11 \times 10^6$ spores/g, respectively and was statistically at par with each other.

At 75 days after inoculation, significantly the maximum spore count ($9.28 \pm 0.14 \times 10^6$ spores/g) was again observed in talc-based formulation with a spore reduction of 45.54 per cent from 15 days after inoculation. However, this trend changed at 90 days after inoculation with the maximum spore count ($6.38 \pm 0.11 \times 10^6$ spores/g) in alginate-based formulation with a spore reduction of 46.24 per cent. Significantly least spore count was recorded in pesta grain-based formulation with $4.35 \pm 0.06 \times 10^6$ spores/g and $2.75 \pm 0.06 \times 10^6$ spores/g and with a spore reduction of 62.34 per cent and 72.19 per cent, respectively at 75 and 90 days after inoculation.

In present study, all the formulations showed a fairly good number of spores even at 90 days after storage. However, talc and alginate-based formulations performed well throughout the study by sustaining the maximum number of spores even upto 90 days after storage. Moreover, the data pertaining to the per cent reduction in spore count revealed that there was a decreasing trend of population from initial day of counting. However, the least spore reduction (58.33%) was observed in alginate-based formulation. This may be due to the fact that sodium alginate is a natural polysaccharide derived from seaweed and it has excellent film-forming and encapsulation abilities. These properties make it an ideal candidate for protecting and prolonging the viability of spores from biotic and abiotic stresses (Bhai, 2020).

The result of the current study is more or less similar to the results of the study conducted by Paramasivan *et al.* (2019) who observed that *T. viride* and *T. harzianum* showed the maximum level of population in talc-based formulation at the end of 120 days after storage. They also recorded that the population of both the fungi showed a decreasing trend from the initial population in all carrier materials except talc-based formulation which showed increasing trend upto 20 days after storage and decreased afterwards.

Cost of Production of Formulations. The approximate cost of production of each formulation used in this study is worked out and presented in the Table 2. The cost which needs to be incurred on the materials required for the formulation, expense spend on labour, electricity usage for autoclaving, inoculation room *etc.*, were taken into consideration.

The approximate cost of production of talc-based formulation was Rs. 456.00/kg which was almost equal to the pesta grain-based formulation which was Rs. 460.00/kg. For vermiculite wheat bran-based formulation, the approximate cost needs to be incurred was Rs. 629.76/kg. The costliest formulation was alginate-based formulation with cost of production of Rs. 635.02/kg.

From this parameter it can be clearly noted that talc-based formulation was not only the cheapest but also easily available formulation. Pesta grain-based formulation was also equally cost effective as talc-based formulation. But its ability to sustain *C. militaris* was found to be poor from our study. Though alginate-based formulation performed equally good as talc-based formulation in sustaining *C. militaris* over a storage period of 90 days, the cost of production of this formulation would be higher. From these assumptions, it could be noted that talc-based formulation would be the cost effective and long-lasting formulation for sustaining *C. militaris.*

Table 1: Spore count of *C. militaris* on various carrier materials at different days after inoculation.

Treatments	Spore count ($x10^6$ spores/g)					
	15 DAI	30 DAI	45 DAI	60 DAI	75 DAI	90 DAI
Talc	17.03 ± 0.25^a (4.13±0.03)**	15.35 ± 0.49^a (3.92±0.06)	14.10 ± 0.44^a (3.75±0.06)	11.10 ± 0.32^a (3.33±0.05)	9.28 ± 0.14^a (3.05±0.02)	5.50 ± 0.09^b (2.34±0.02)
Vermiculite wheat bran	12.23 ± 0.24^c (3.50±0.03)	10.70 ± 0.30^c (3.27±0.05)	9.18 ± 0.17^c (3.03±0.03)	6.73 ± 0.11^c (2.59±0.02)	4.83 ± 0.13^c (2.20±0.03)	3.05 ± 0.06^c (1.75±0.02)
Pesta grain	11.55 ± 0.12^c (3.40±0.02)	9.18 ± 0.06^d (3.03±0.01)	8.00 ± 0.25^d (2.83±0.04)	6.20 ± 0.11^c (2.49±0.02)	4.35 ± 0.06^d (2.09±0.02)	2.75 ± 0.06^d (1.66±0.02)
Alginate	15.30 ± 0.31^b (3.91±0.04)	14.13 ± 0.21^b (3.76±0.03)	12.65 ± 0.17^b (3.56±0.02)	10.48 ± 0.19^b (3.24±0.03)	8.23 ± 0.13^b (2.87±0.02)	6.38 ± 0.11^a (2.52±0.02)
C.V. (%)	3.42 (1.73)	4.94 (2.36)	5.11 (2.48)	4.66 (2.20)	3.56 (1.79)	3.85 (1.98)

Data are means± standard error of means of four replicates

**Figures in parenthesis are square root transformed values

Values in columns followed by the common letter(s) are not significantly different at 5% level of significance analysed using Least Square Difference method

Table 2: Cost of production of different formulations of *C. militaris.*

Sr. No.	Carrier	Materials required (kg)	Price (Rs.)	Miscellaneous expenses (Rs.)	Total cost of production (Rs./kg)
1.	Talc	Talc-1 kg	25.00	*CMC- Rs. 1 (5gram) Packaging materials (1 piece)- Rs. 10 Labour-400/day Electricity-20/day	456.00
2.	Vermiculite wheat bran	Vermiculite (0.752 kg)	150.40	Packaging materials (1 piece)- Rs. 10 Labour-400/day Electricity-20/day	629.76
		Wheat bran (0.248 kg)	48.36		
3.	Pesta grain	Wheat flour (1 kg)	30.00	Packaging materials (1 piece)- Rs. 10 Labour-400/day Electricity-20/day	460.00
4.	Alginate	Sodium Alginate (0.334 kg)	75.15	Packaging materials (1 piece)- Rs. 10 Labour-400/day Electricity-20/day	635.02
		Wheat bran-(0.666 kg)	129.87		

*CMC- Carboxy Methyl Cellulose

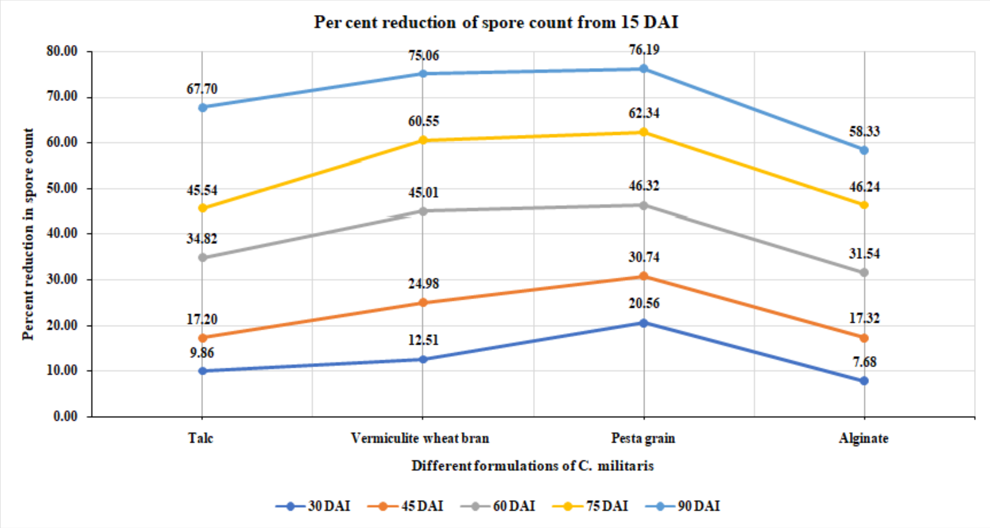

Fig. 1. Per cent spore reduction in different carrier based formulations of *C. militaris.*

CONCLUSIONS

From the current investigations, it can be clearly observed that the talc-based formulation and alginate-based formulation performed well at all the days of observation and had a fairly good spore count even at 90 days after inoculation. However, talc-based formulation had a vantage over alginate-based formulation by having a less production cost.

FUTURE SCOPE

As *Cordyceps militaris* have both insecticidal and anti-microbial properties, the fungus can be used as a part of integrated pest management practise to manage both insects and diseases.

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Conflict of Interest. None.

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