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Effect of Temperature and Storage Time on Shelf Life of Mycoherbicidal Products of *Colletotrichum dematium* FGCC#20

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Abstract: Problem statement: The storage environment can have a drastic impact on the stabilization of microbial biopesticides. As a general rule, storing microbial preparation at lower temperature increase long term survivelity. **Approach:** Optimization of shelf life is critically important for biocontrol products containing living microorganisms. Conidia of *Colletotrichum dematium*, (FGCC#20) a fungal pathogen of the weed, *Parthenium hysterophorus* were produced in solid agrowaste substrate. After solid based formulation, formulated mycoherbicidal products were conditioned at room temperature and at 4°C temp in different storage periods. **Results:** The longest shelf-life (conidial inoculums viability) was found in formulated products stored at 4°C temperature. At this temperature 4°C pesta (Wheat bran + Keolin) granules were 80% viable for 3 months and 10-15% viable after one month. **Conclusion:** The temperature had a renounced effect on the shelf life of the formulated products during storage. Maximum shelf life was observed in pesta granules for at least 1 year at 4°C would be adequate for commercialization.

Key words: Biocontrol products, *Colletotrichum dematium* formulation, parthenium, mycoherbicide, shelf-life, storage

INTRODUCTION

Parthenium hysterophorus, an alien invasive species, The weed is also responsible for several health problems to human and animal, which has how emerged as one of the world's worst weeds causing enormous losses to crop productivity, biodiversity and environment in India. As conventional strategies for its management have failed exploitation of microorganism and their byproducts as ecofriendly herbicide has generated significant interest world wide (Pandey *et al*., 1996a; 1996b; Jayalakshmi *et al*., 2005).

A bioherbicidal product need an adequate shelf-life to be commercially acceptable as an alternative or supplement to chemical herbicides. Storage conditions specially temperature, moisture and formulations are known to influence the (Sher, 1999; Connick *et al*., 1996; Abbas and Boyette, 1994; 2000; Amsillen *et al*., 1999; Daigle *et al*., 1998; Abbas and Egley, 1996). Potential of *Colletotrichum* spp. is well recognized as evidenced by commercialization of many of them as mycoherbicides (Boyette *et al*., 1993; Cartwright and Templeton, 1992; Templeton, 1992). *C. dematium* FGCC#20 recovered from disease seedling of the forest weed have shown excellent mycoherbicidal properties against following weeds. However, understandings regarding storage conditions are for sustainable use of a bioherbicide. Therefore the present study was undertaken to standardized the storage conditions required for maximum shelf-life of the products.

MATERIALS AND METHODS

Mycoherbicidal agent: *Colletetriehum dematium* (FGCC#20), isolated from diseased seedlings of *Parthenium hysterophorous* L. (Gayathri and Pandey, 1997). The test agent was obtained from Fungal Germplasm Collection Centre (FGCC), Dept. of Biological Science RD University, Jabalpur (MP) India. Lyophilized stock cultures were stored in refrigerator. Inoculam were activated as per (Jakson and Schirler, 1992). Sporulated potato dextrose agar plates were rinsed with sterilized distilled water and conidial suspension @ 5×10^5 conidial mL^{-1} was used as inoculum.

Conidia produced in agrowaste substrate: Agrowaste substrate, maize cob grits were soaked overnight in distilled water. Pretreatment of hardcore substrate were carried out as per (Rodriguez-Vazguez *et al*., 1992),

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moisture of the substrates were 60% respectively. For sterilization 5.0 g of each moistened substrate was placed into a 500 mL Erlenmeyer flask which was thoroughly sterilized at 15 psi for 30 min. Each flask was separately seeded with 5.0 mL spore suspension (5×10^5 conidia mL^{-1}) incubated at 28±1°C in a BOD incubator (Yorco, India) for 15 days.

Determination of moisture quantity in substrate: Before inoculation and after autoclaving substrates were removed under laminar airflow. Then these substrates were weighed (1 g) and kept into the hot air oven Yorco at 50°C for 24 h for drying. After 24 h all the substrates were removed from the hot air oven and weighed further and moisture quantity of different substrate were calculated by using following formula.

$$\text{Moisture quantity (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Where:
Initial weight = 1 g of substrate after autoclaving
Final weight = Weight of dried substrate

Extraction of conidia: About 10 g of each infested substrate was weighed, dried crushed with the help of sterile pestle and mortar and then mixed in to the 1000 mL of distilled water. The spore suspension was filtered thorough cheese cloth and 1 mL of Tween 80 added per 1000 mL of spore suspension as an adjuvant. Spores Load Per Gram (SPLG) was estimated by using following formula (Bharadwaj and Trivedi, 1998):

$$\text{SPLG} = \frac{N \times V \times 1000}{W}$$

Where:
N = No. of spores in centre square of haemocytometer
V = Volume of mounting fluid added to the substrate
W = Weight of the substrate

Storage of formulated products in different temperature:

Kaolin telecom powder: The Kaolin telecom powder was sterilized and mixed with an aqueous suspension of spores (1 g of Kaolin per 10^9 spores) grown on PDA plates. This mixture was then centrifuged for 15 min at 300 rpm. The pellet was air dried under a constant flow of sterile air overnight, pulverized using a pestle placed in glass tubes and sealed. Samples were stored at 20°C or 4°C. The percentage of water content in the dried kaolin-spores mixture was 10%.

Prepared samples of kaolin spore mixture were stored in different temperature. The percentage of water content ion the dried Kaolin-spore mixture was 10%. To determine spore viability and virulency of different time interval were studied (Norman and Trajillo, 1995).

Pesta formulation: The pathogen grown was homogenized, added to mixture of wheat straw and wheat flour and kneaded sheets and dried under the sun light for 2 days. After drying the sheets were ground or broken and sieved were ground of 1.2 mm. Sustained conidial production and viability of the pesta granules were tested periodically by plating on PDA plates. Three pesta granule combinations $pesta^a$ (wheat bran + kaolin + spore suspension), $pesta^b$ (wheat flour + spore suspension), $pesta^c$ (gram flour + spore suspension) (Connick *et al*., 1996; 1994). were prepared. Dried pesta were placed in zip-lock bags and stored in different temperatures (28 and 4°C).

Alginate granules: About 100 mL spore suspension (15×10^5 conidia mL^{-1}) were mixed into a 8% sodium alginate (100 mL solution that may also contain an inert filter (Kaolin) and nutrients (Soya flour or corn flour) and make 1 M solution of $CaCl_2$, which was kept on magnetic stirrer, where the calcium ions react with the sodium alginate which contain spore suspension to transform to each droplet into a gel bead (calcium alginate). The gel beads were removed after few minute residence times in the gelatin bath and air dried in Laminar air flow (Paul *et al*., 1993), drying convents the gel beads in to hard granules fairly uniform and size. Alginate granules were placed in zip-lock bags and stored in 28 and 4°C.

Fungus infested dry substrate: Properly colonized substrates were winged and dried through hot air blower at 22°C sterile conditions, dried substrate packed in seal packed polythene bag and stored at 4°C and room temperature. Dried infected substrates were stored at 4°C and room temperature in a zip-lock polythene bags. Viability and virulency of all formulated mycoherbicidal products were checked in particular time intervals.

Sampling and assay of products (viability tests): About 1 g sample of formulated products were suspended in 100 mL sterile distilled water centrifuged at 8000 rpm for 15 min and supernatants were discarded. The pellets were resuspended in 100 mL of sterile distilled water, homogenized and serial dilutions were prepared. Each sample was plated on Potato Dextrose Agar (PDA) medium and cfu (colony forming units)

were estimated after 7 days of incubation (Couteaudier and Alabiuvette, 1990). All the formulated products were divided into two sets and one set A was stored at room temperature (28°C) and set B was stored at 4°C. Samples were taken out after one-month interval were serially diluted and plated on PDA for cfu[s] (colony forming unit) count.

Seedling bioassay technique (virulency tests): Efficacy of various formulated products were evaluated though seedling bioassay. It was performed as accordance with Berger and Hanson (1963); Waston and Winder (1994); Sukumar and Ramalingm (1981) and Makowski (1993). Two weeks old Parthenium seedlings grown in the plant growth chamber Yorco were sprayed to run off with conidial suspension. The necessary saturated atmospheres following inoculation were obtained by placing the plants in an environmental test chamber Remi (28±1°C and 90% RH). Control seedlings were sprayed to run off with distilled water only. Treated as well as control sets were observed daily for symptoms and the development of the diseases, percent seedling mortality was recorded up to 7 Day After Treatment (DAT).

Statistical analysis: All experiments were carried out in triplicates. Representative data were subjected to Analysis of variance and means of the various treatments were separated by fisher's Least Significant Difference (LSD) (SAS Institute, 1988).

RESULTS

It is evident from the data recorded during the present investigation that viability and virulency of conidia of *C. dematium* (FGCC#20) varied significantly amongst various formulations (Table 1 and 2 and Fig. 1 and 2). Maximum cfu's (75-95%) were recorded in all pesta preparations (pesta a, b, c) stored for 90 days at 4°C. It was followed by Kaolin telecom powder (65-85%), Alginate granules (60-75%), fungus infested dry substrate (58-70%) and substrate Kaolin powder (50-60%). After 180 days again it was observed that more than 25% conidia survived in the pesta preparation (a, b, c) granules (20%). It is followed by substrate kaolin powder (10%), Alginate granules (5-6%), Kaolin telecom powder (6%) and Fungus infested dry substrate (4%). Population of the fungus declined gradually after 240 days in all formulated products (Table 1).

It is observed that at room temperature (28±1°C) all formulated products declined gradually in day by day formulation viz., Kaolin telecom powder and fungus infected dry substrate stored at room temp. whereas in rest of the formulation viz., Pesta granules a, b and c and Alginate granules, the number of colonies increases up to 180 days and declined there after (Table 2) At room temperature (28±1°C). Pesta granules containing conidia retained (85-35%) within 180 days. There after the viability declined sharply in pesta granules containing conidia, reaching only 2 and 1% after 240 days.

Virulence of the pathogen was found to be highly variable under different storage condition. The seedling mortality was maximum for longer time when the fungus stored at lower temperature (4°C) Fig. 1. Maximum seedling mortality was observed in all formulated products in 0 days of storage or fresh products was 82% in kaolin talc, 87, 82 and 79% in pesta granules a b c, 90% in alginate granules, 92% in fungus infected dry substrate and 67% in Kaolin substrate powder. The effectiveness drastically declined in all formulated products. Maximum seedling was observed in fungus infected dry substrate and Alginate granules. Poor seedling mortality was observed after 240 days in all products when stored in 4°C.

Table 1: Shelf life of *C. dematium* (FGCC#20) in different formulated products at 4°C temperature

	Population of *C. dematium* (cfus) at 4°C temperature				
Formulated products	30 days	90 days	180 days	240 days	270 days
Talc (Kaolin)	90.00±1.00	83.67±1.53	75.67±1.00	16.00±1.00	10.00±1.00
Pesta[a] granules	97.67±2.65	91.00±1.00	85.00±1.00	44.33±2.08	27.67±1.53*
Pesta[b] granules	92.00±2.00	96.67±1.53	81.67±2.08	40.00±2.00	24.33±1.53
Pesta[c] granules	98.33±1.53	90.33±1.53	84.33±2.08	45.33±2.08	24.33±0.58
Alginate granules	93.33±2.08	92.00±2.00	84.33±2.08	55.67±1.53	27.62±1.50*
Fungus infested dry substrate	85.67±3.21	84.67±3.51	81.33±1.53	30.00±1.00	12.00±1.00

Table 2: Shelf life of *C. dematium* (FGCC#20) in different formulated products at room temperature

	Population of *C. dematium* (cfus) at room temp				
Formulated products	30 days	90 days	180 days	240 days	270 days
Talc (Kaolin)	85.33±2.52	75.00±1.00	35.67±2.08	15.67±2.08	5.00±1.00
Pesta[a] granules	87.33±2.08	74.67±1.53	41.33±1.53	22.67±2.52*	1.67±1.53
Pesta[b] granules	89.00±1.00	76.67±1.53	32.00±2.00	12.00±2.00	1.00±1.00
Pesta[c] granules	84.33±1.53	74.00±2.00	31.00±1.00	20.67±2.08	1.52±2.52
Alginate granules	86.67±1.53	75.67±2.08	25.67±2.52	22.35±1.53*	0.00±0.00
Fungus infested dry substrate	87.33±2.08	78.00±2.65	27.67±1.53	22.33±0.58	1.16±1.53

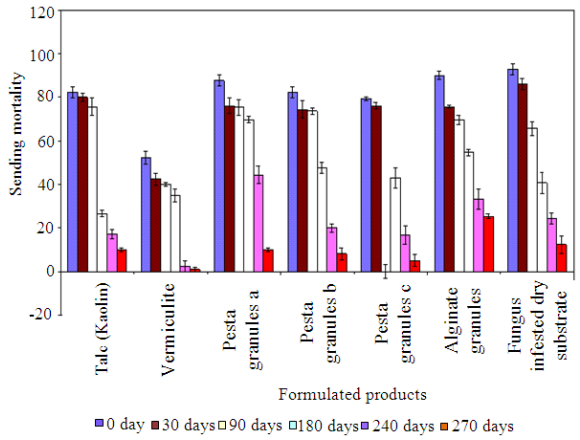

Fig. 1: Virulence of *C. dematium* (FGCC#20) in different formulated products at 4°C temperature

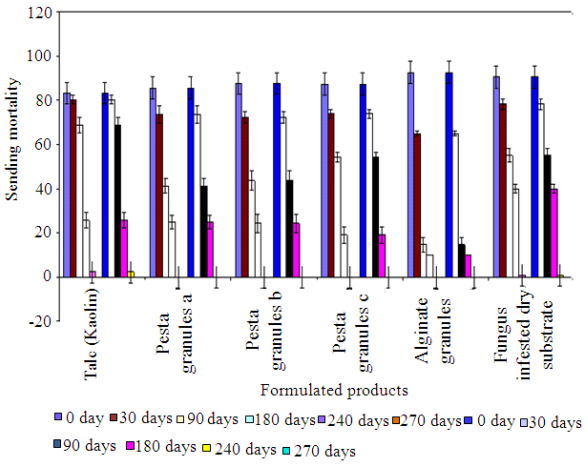

Fig. 2: Virulence of *C. dematium* (FGCC#20) in different formulate products at room temperature (28°C)

At room temperature (28°C) maximum seedling mortality was observed in alginate granules and fungus infected dry substrate. Poor seedling mortality rate was observed in case of room temp (25±1°C) after 90 days in all products. At higher temperature and humidity the loss of virulence was significant in all the formulated products. After 240 days seedling mortality was observed in only alginate granules and fungus infested dry substrate Fig. 2. From the stand point the inoculum survival in all formulated products, pesta granules a, b, c and Alginate granules were the most suitable formulation. Since it showed high stability over time the present study.

DISCUSSION

A Similar result has been reported (Elzein *et al*., 2001). A formulation that contains the biological agent in a state of low metabolic activity makes the agent more tolerant to environmental stresses such as temperature fluctuations and extremes and humidity stress and tends to support a longer shelf-life (Paau, 1998). This experiment can be explain the longer viability of conidia in formulated products during storage.

Although the efficiency or virulency of the products was also tested (Connick *et al*., 1996), showed the capability of 18 month old granules stored in the refrigerator to cause more then 50% infection and killing of the target weed. A similar result was obtained when 6 month-old pesta granules containing dried chlamydospores rich biomass of *F. oxyrporum* F. sp. Orthocers stored at room temp were incorporated in to the soil to control the parasitic weed *Orbamehe cumana* Wallr (Stover *et al*., 2004).

CONCLUSION

In conclusion the temperature had a renounced effect on the shelf-life of the formulated products during storage. Maximum shelf-life was observed in pesta granules and Alginate granules for at least 1 year at 4°C would be adequate for commercialization. Moreover, the viability of formulated products during the first 6 months at room temperature would be sufficient for its use under realistic conditions of storage, humidity and delivery.

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