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Effect of storage conditions on the survival of two potential biocontrol agents of nematodes, the fungi *Paecilomyces lilacinus* and *Pochonia chlamydosporia*

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The nematophagous fungi *Paecilomyces lilacinus* and *Pochonia chlamydosporia* have been extensively studied as biological control agents for plant-parasitic nematodes. This study describes the formulation of alginate pellets containing mycelia of these fungi and also describes the effect of storage conditions on shelf-life of the pellets. The shelf-lives of *P. lilacinus* and *P. chlamydosporia*, which were measured monthly for 6 months, were significantly improved at low temperatures and low water activity (a_w) values (<0.33). Vacuum did not affect the viability of the formulated *P. lilacinus* but increased the viability of *P. chlamydosporia*. Carbon dioxide reduced the activity of *P. lilacinus* as compared to ambient air but increased the activity of *P. chlamydosporia*. Nitrogen, however, significantly improved the viability of both fungi. The optimal parameters of each factor for our formulation of *P. lilacinus* and *P. chlamydosporia* included a temperature range of 4 to -20°C, $a_w = 0.12$, and a nitrogen-filled atmosphere.

Keywords: formulation; *Paecilomyces lilacinus*; *Pochonia chlamydosporia*; shelf-life; storage conditions

Introduction

The use of fungal biological control agents that have been formulated for inundative application has been extensively studied but only a few products are commercially available (Liu and Li 2004). Two of the major reasons are the short shelf-life of the fungal formulations and the high cost of mass production. Many factors affect the shelf-life of fungi formulated as biological control agents. These factors include active ingredients (Connick, Jackson, Williams, and Boyette 1997; Elzein, Kroschel, and Müller-Stöver 2004b), additives (Jackson, Erhan, and Poprawski 2006; Guijarro, Melgarejo, and De Cal 2007), drying process (Larena, De Cal, Liñán, and Melgarejo 2003), and storage conditions (Connick, Daigle, Boyette, and Williams 1996; Elzein, Kroschel, and Müller-Stöver 2004a; Hong, Edgington, Ellis, de Muro, and Moore 2005; Friesen, Holloway, Hill, and Pugsley 2006). The most important environmental factors affecting shelf-life are temperature and water activity (Connick et al. 1996; Hong et al. 2005). A number of studies demonstrated that viability of some formulated fungi was poor at room temperature (Lawrie, Down, and Greaves 2001; Elzein et al. 2004a; Guijarro et al. 2007). The atmosphere in the package may also be important for some formulations (Friesen et al. 2006; Teshler, Ash, Zolotarov, and Watson 2007).

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For fungi formulated as mycopesticides, the stage most commonly used as the 'active ingredient' is the spore (Faria and Wraight 2007). For example, formulations of *Beauveria bassiana* and other entomopathogenic fungi usually contain conidia produced by first growing mycelia in liquid medium and then inducing sporulation on a solid substrate (Feng, Poprawski, and Khachatourians 1994). Sporulation on a solid substrate, however, generally requires much time and space and is often cost ineffective (Jenkins, Heviefo, Langewald, Cherry, and Lomer 1998; Ye, Ying, Chen, and Feng 2006). Separation of fungal spores from the mycelia can also be difficult (Leštan, Leštan, and Lamar 1998).

In contrast to the production of spores on solid substrates, production of mycelia and submerged spores by liquid fermentation can result in high yields in a relatively short time (Hofstein and Chapple 1999). Although biomass production of mycelia and submerged spores is more economical than production of spores, the short shelf-life of fungal formulations based on mycelial biomass is one of the obstacles to commercial use of biocontrol fungi.

The nematophagous fungi *Paecilomyces lilacinus* and *Pochonia chlamydosporia* have long been recognised as potential biocontrol agents against plant-parasitic nematodes (Chen and Dickson 2004; Kiewnick and Sikora 2006). Previous researchers, however, have paid more attention to screening isolates and evaluating efficacy than to improving fungal shelf-life (Stirling and Smith 1998; Verdejo-Lucas, Sorribas, Ornat, and Galeano 2003). In this study, we used the methods of Walker and Connick (1983) to develop alginate pellet formulations containing mycelia of these two fungi. Our purpose was to determine the effect of different storage conditions (temperature, water activity, and atmosphere) on the survival of the formulated *P. lilacinus* and *P. chlamydosporia*.

Material and methods

Fungal isolates

Paecilomyces lilacinus YES-2 and *P. chlamydosporia* HDZ-9 were isolated from eggs of the root-knot nematode and deposited in the China General Microbiology Culture Collection as CGMCC NO.2012 and CGMCC NO.310073. Both isolates were highly parasitic to the eggs of the root knot nematode during screening (Sun, Gao, Shi, Li, and Liu 2006). All investigations were performed with a single-spore isolate of each fungus.

Pellet formulation

Alginate pellets containing mycelia of the fungi were prepared using the methods of Walker and Connick (1983) with some modifications. To obtain mycelia-rich biomass, YES-2 and HDZ-9 were cultured in different liquid media (Sun et al. 2006) on a rotary shaker (150 rpm) at 25°C for 5 days. Fungal mycelia were harvested from the liquid culture by filtration with four layers of paper towel. The fungal inocula were prepared by homogenising the fungal mycelia in 500 mL of sterile distilled water with a mini-sample blender for 60 s at high speed. The suspensions were then mixed with 500 mL of 4% sodium alginate solution containing diatomite clay (Beijing Chemical Reagents Company, Beijing, China); the final concentration of the diatomite clay was 10% (w/v). The sodium alginate solution and diatomite clay were autoclaved before use, and all operations were carried out on the clean bench. Streptomycin at 100 ppm and chloramphenicol at 50 ppm were added to the mixtures to prevent bacterial contamination. After the mixtures were placed on a shaker at 200 rpm for 5 min to thoroughly mix the components, they were placed in a container with 2-mm diameter aperture openings in the bottom (50 mL). The drops that fell from the container were collected in a beaker containing a sterile 0.2 M calcium chloride solution. When the alginate mixture contacted the calcium chloride solution, pellets formed immediately. These pellets were then collected on a sieve and washed three times with sterile distilled water to prevent them from sticking together. The washed pellets were air-dried for 4–5 days at 30°C in a dryer before testing for viability. Water contents of the dried pellets containing *P. lilacinus* and *P. chlamydosporia* were 7.44 and 6.71%, respectively.

Viability test

The shelf-life (viability after storage) of the pellets was determined at 1-month intervals for 6 months using the methods described by Lee and Heo (2000) with some modifications. For each isolate, 10 random samples of alginate pellets were weighed and then agitated in a 50-mL centrifugal tube containing 40 mL of 0.1 M citrate sodium for 3 h. The resulting suspensions were vigorously mixed using a vortex mixer. Serial dilutions were prepared, and 0.2 mL of each homogenised suspension was added to a PDA plate supplemented with 300 ppm streptomycin and 100 ppm chloramphenicol. The dilution plates were incubated for 4 days at 25°C until colonies were visible. The colony forming units (CFUs) were counted after 4 days at 25°C, and the data were expressed as CFU g^{-1} of pellet. The initial viability of pellets was assessed after the pellets were dried. Each experiment had three replicates per treatment and was repeated three times.

Effect of temperature on viability during storage

After pellets were air dried, 2-g samples (about 180 pellets for YES-2 and 140 for HDZ-9) were placed in 15-mL plastic centrifuge tubes and sealed with Parafilm. The tubes were placed in incubators at −20, 4, 25, or 40°C. At monthly intervals during storage, 10 pellets were removed from each centrifuge tube, and viability was assessed as described above.

Effect of a_w *on viability during storage*

After pellets were air dried, 2-g samples were placed in 50-mL centrifuge tubes, which in turn were placed in triangular flasks at 25°C. A hole had been formed in the upper wall of each centrifuge tube to permit equilibration of the a_w in the tube and the flask. The a_w in each flask (and tube within) was adjusted to 0.12, 0.33, 0.53, and 0.75 by placing saturated solutions of lithium chloride, magnesium chloride, magnesium nitrate, and sodium chloride, respectively, in the flask bottoms (Winston and Bates 1960; Greenspan 1977). The flasks were covered with two layers of polycarbonate membrane with bacterial filter and sealed with Parafilm to prevent water evaporation and contamination. Pellets were sampled and tested for viability as described above.

Effect of storage atmosphere on viability

After pellets were air dried, 1-g samples were placed into Hungate Tubes (Attebery and Finegold 1969). The Hungate Tube had a 1-cm diameter, 15-cm long cylinder with a double gasket screw top. For incubation of pellets in the ambient atmosphere, samples were directly placed in a Hungate Tube sealed with Parafilm to improve the seal. For other treatments, a 0.1-mm diameter syringe was inserted into the gasket, and the tube was deflated with a pump for 10 s. The tube was then filled with pressurized nitrogen or carbon dioxide for 10 s or was treated with vacuum for 10 s. The procedure was repeated eight times. After the last addition of nitrogen or carbon dioxide, or after the last application of vacuum, the syringe was quickly withdrawn and the tubes were sealed, leaving tubes with ambient air, nitrogen, carbon dioxide, or vacuum (reduced atmospheric pressure). Subsamples were taken at monthly intervals from each of the four treatments to determine viability as described above. Each time the tubes were opened and subsamples were removed, nitrogen, carbon dioxide, or vacuum treatments were re-established using the same procedures described earlier in this paragraph.

Statistical analysis

Analysis of variance was carried out using SPSS 13.0 statistical package. Viability data (number of colony forming units (CFU) g^{-1} of formulation) were transformed into log and subjected to analysis of variance (ANOVA). Means and standard deviations were calculated, and when F values were significant at $P < 0.05$, means were separated by the Student–Newman–Keuls method.

Results

Pellet formulation

The dried pellets prepared were spherical and of uniform size for both fungi. However, the size and weight were somewhat different according to the fungus. The pellet diameter and weight were 2.82 ± 0.21 mm and 11.43 ± 0.56 mg for *P. lilacinus* YES-2, and 3.46 ± 0.32 mm and 14.47 ± 0.93 mg for *P. chlamydosporia* HDZ-9.

Effect of storage temperature on viability

The viability of the two nematophagous fungi YES-2 and HDZ-9 declined very little when stored for up to 6 months at 4 or −20°C but declined rapidly when stored at 25 or 40°C (Figure 1). The viability did not statistically differ at 4 or −20°C.

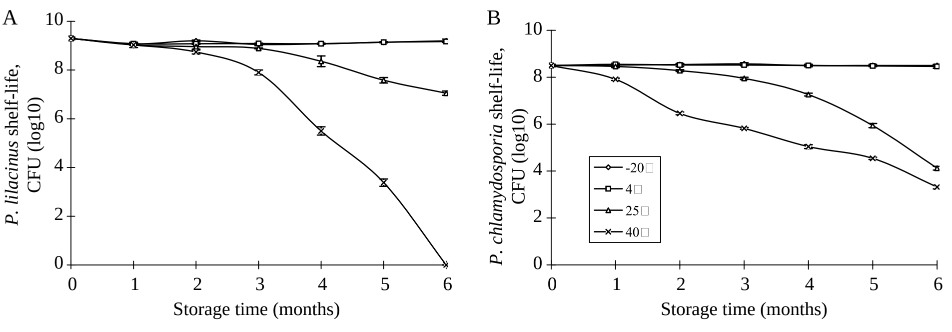

Figure 1. Effect of temperature (40, 25, 4, and −20°C) on the survival of the formulated fungi (A) isolate YES-2 of *P. lilacinus* and (B) isolate HDZ-9 of *P. chlamydosporia*. Viability data (CFU g^{-1} of formulation) were log transformed. Each value is the mean (±95% confidence intervals) of three replications averaged over three trials of the experiment.

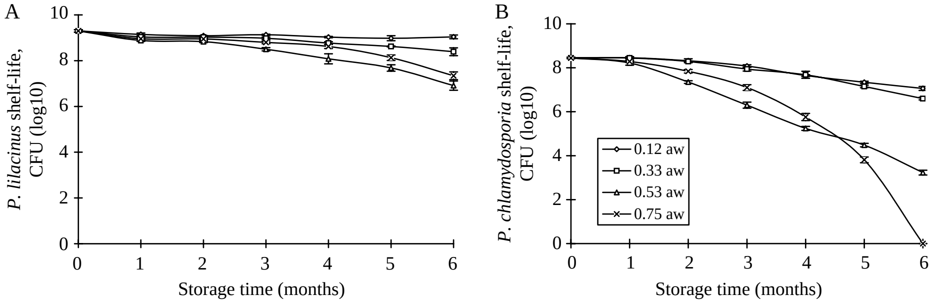

Figure 2. Effect of constant water activities (0.12, 0.33, 0.53, and 0.75 a_w) on the survival of the formulated fungi (A) isolate YES-2 of *P. lilacinus* and (B) isolate HDZ-9 of *P. chlamydosporia*. Viability data (CFU g^{-1} of formulation) were log transformed. Each value is the mean (±95% confidence intervals) of three replications averaged over three trials of the experiment.

Effect of storage a_w on viability

The viability of both fungi declined substantially at higher a_w values (0.53 and 0.75) but remained remarkably high at 0.12 and 0.33 a_w (Figure 2). In other words, viability tended to remain high under drier conditions but declined under wetter conditions.

Effect of storage atmosphere on viability

For stored pellets of YES-2, viability did not significantly differ with vacuum and ambient air but was significantly higher with nitrogen and lower with carbon dioxide. For stored pellets of HDZ-9, viability was highest with nitrogen, lowest with ambient air, and intermediate with carbon dioxide and vacuum (Figure 3).

Discussion

This study has shown that viabilities of pellets of the nematophagous fungi *P. lilacinus* and *P. chlamydosporia* were markedly influenced by storage temperature, a_w, and atmosphere. In general, pellets were much more stable when stored at 4 or −20°C than at 25 or 40°C. Similar observations have been noted by Wu and Hsiang (1998), who reported that

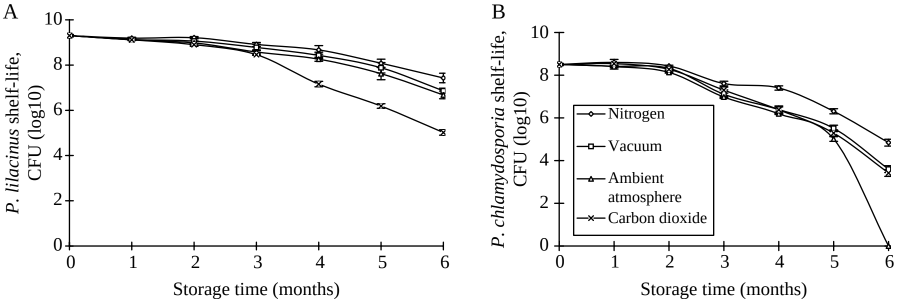

Figure 3. Effect of different atmospheres (nitrogen, vacuum, ambient atmosphere, and carbon dioxide in the package) on the survival of the formulated fungi (A) isolate YES-2 of *P. lilacinus* and (B) isolate HDZ-9 of *P. chlamydosporia*. Viability data (CFU g^{-1} of formulation) were log transformed. Each value is the mean (±95% confidence intervals) of three replications averaged over three trials of the experiment.

viability of pelletised alginate formulations of *Typhula phacorrhiza* remained high at low temperature but declined substantially at high temperature. The shelf-life of the biocontrol agent *Colletotrichum truncatum* (in 'Pesta formulation') was also greater at 4°C than at higher temperatures (Connick et al. 1996). The longer shelf-life at low temperature could be attributed to the lower metabolic activity of the fungal propagules (Elzein et al. 2004a). Moreover, temperatures above the optimum for growth generally represent a stress to fungi (Leong, Hocking, and Scott 2006). For *P. lilacinus* and *P. chlamydosporia*, optimal growth occurs at or below 30°C (Olivares-Bernabeu and López-Llorca 2002; Kiewnick 2006), and so cold storage may be required to maintain the viability of these formulations. The high cost of refrigeration would be a major limiting factor for a commercial product (Honeycutt and Benson 2001). Our results, however, indicate no significant difference in viability with storage at 4 or −20°C for both fungi. Obviously, it is more economical to store formulations at 4 than −20°C, and this finding suggests that maintaining a suitable storage temperature could be economical.

Our results also demonstrated a strong effect of constant a_w on the survival of formulated *P. lilacinus* and *P. chlamydosporia* at 25°C. Throughout the storage period, viability was always higher when pellets were stored at 0.12 a_w rather than 0.75 a_w. The fungal shelf-life was extended at 0.12 and 0.33 a_w. These results were consistent with those obtained with *C. truncatum* in Pesta formulation (Connick et al. 1996) and with *Rhizoctonia* spp. in Pesta and rice flour formulations (Honeycutt and Benson 2001). Low a_w (dry conditions) may increase survival by keeping the organisms in a state of low physiological activity. Surprisingly, survival was greater at 0.75 a_w than at 0.53 a_w after 6 months storage for *P. lilacinus* and during 1–4 months for *P. chlamydosporia*. This phenomenon has been also noted in other studies (Connick et al. 1996).

Composition of the storage atmosphere is another important factor affecting the viability of stored fungi (Guynot, Marin, Sanchis, and Ramos 2003; Teshler et al. 2007). The gases commonly used in packaging formulations are nitrogen, carbon dioxide, and oxygen (Abellana, Ramos, Sanchis, and Nielsen 2000). A nitrogen-rich environment limits oxygen uptake and thereby slows fungal metabolism, limits oxidative reactions, and lacks antifungal properties (Abellana et al. 2000). The viabilities of our *P. lilacinus* and *P. chlamydosporia* formulations were generally higher when stored in a nitrogen atmosphere than in a carbon dioxide atmosphere, an ambient air atmosphere, or a vacuum.

In this study, we examined the independent effects of temperature, a_w, and atmosphere on storage of two nematophagous fungi. Other research, however, has shown that these and related factors may not act independently. For example, Farber (1991) found that carbon dioxide had a fungistatic effect, which depended on many factors including the partial pressure of carbon dioxide, oxygen concentration, volume of headspace gas, temperature, acidity, and a_w. Magan and Lacey (1984) also demonstrated that storage of fungi under low oxygen or high carbon dioxide concentrations was highly dependent on a_w and temperature. It should be emphasised that the influence of each factor on the survival of a specific fungal isolate should be determined on a case-to-case basis. The interactions of different storage gases, temperature and a_w should be investigated in detail. The practical use of biological control products could also be increased by new information about the effects of modified atmosphere packaging (MAP) on the shelf-life of food (Fonseca, Oliveira, and Brecht 2002).

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