Scientific Research Publishing

American Journal of Plant Sciences, 2017, 8, 3272-3284
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Extending the Shelf-Life of *Myrothecium verrucaria*, a Bioherbicide

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How to cite this paper: Hoagland, R.E., Boyette, C.D. and Stetina, K.C. (2017) Extending the Shelf-Life of *Myrothecium verrucaria*, a Bioherbicide. *American Journal of Plant Sciences*, **8**, 3272-3284.
https://doi.org/10.4236/ajps.2017.812220

Received: October 25, 2017
Accepted: November 25, 2017
Published: November 29, 2017

Abstract

The shelf-life of a bioherbicide product is an important factor with regard to its commercial potential. The bioherbicidal efficacy of freshly fermented *Myrothecium verrucaria* (strain IMI 368023) (MV) mycelia formulations and MV mycelia preparations that had been freeze-dried and then stored at −20˚C for 8 years was compared. Two concentrations of each formulation (1.0x and 0.5x) were tested, utilizing bioassays on seedlings of the weed, hemp sesbania (*Sesbania exaltata*) under greenhouse conditions or in darkness utilizing hydroponically grown seedlings. Freeze drying of freshly prepared MV mycelium produced a light, brownish-colored powder. Efficacy tests of this reconstituted 8-year-old dried material showed that some bioherbicidal activity was lost during long-term storage, *i.e.*, ~20% and ~60% seedling dry weight reduction at the 1.0x and 0.5x rate, respectively. Although plant mortality was greater in the fresh mycelial preparations treatments versus the freeze-dried and stored samples at all time points in the time-course, the stored material still caused >80% mortality, 15 days after treatment. Comparative disease progression ratings also showed a similar trend. Overall results show that freeze-drying MV is a useful method to reduce the bulk and cumbersomeness of storing heavy liquid fermentation product, while retaining bioherbicidal activity. These findings increase the utility of this bioherbicide and offer the potential to use the dried material in soil treatments or in a more concentrated form than attainable via the fermented product.

Keywords

Bioherbicide, Plant Pathogen, Weed Control, Lyophilization, Freeze-Drying, Mycelium, Mycoherbicide, *Myrothecium verrucaria*

1. Introduction

The world's population of ~7.3 billion could reach 9.7 billion by 2050 and 11.2 billion by the year 2100 [1], which in turn will require a dramatic increase in food production. Pesticides are pivotal to maintaining and protecting food crops and food products, with herbicides as the major pesticide class (~50%) with respect to sales. The global herbicide market is predicted to grow to $31.5 billion by 2020 [2]. Although billions of dollars are spent annually on herbicides, numerous additional costs are also associated with weeds (e.g., herbicide application, cultivation, damage to equipment, reduced crop yields due to weed competition, and reduced crop price from weed seed contamination). Synthetic herbicides have been a boon to weed control in agriculture and in other areas, however there are still many weed control problems and many weeds have developed resistance to herbicides [3].

An alternative to synthetic herbicides for weed control is the concept of bioherbicides. Bioherbicides are microbes and/or microbial phytotoxins used to control weeds. This concept gained a lot of impetus during the 1970s and much research has been carried out as evidenced in books [4] [5] [6] and reviews [7]-[18] on bioherbicides. Several general inherent issues arise when these living agents (mostly fungal and bacterial phytopathogens) are utilized as weed biocontrol agents, or when they are being developed as marketable, commercial products. These issues include: efficacy, host specificity, formulation, production, maintaining free moisture (or dew) to support propagule germination and infection on target weeds, coping with hostile environmental field conditions, and storage and shelf-life of products. All of these factors and others are important.

With regard to storage and shelf-life, several methods have been utilized to stabilize or prolong the activity of certain potential bioherbicides, either to maintain microbial cultures, or to stabilize the bioherbicidal product and extend storage time. Generally, cultures have been maintained on potato dextrose agar (PDA) and transferred periodically, with occasional re-inoculation and re-isolation from the host plant. Cultures have also typically been preserved on sterilized soil for long-term storage [19] [20].

For long-term storage of formulations or products, other considerations have prevailed. For example, the shelf-life of conidia and mycelium of two bioherbicides, *Fusarium arthrosporioides* and *F. oxysporum*, was enhanced with a mixture ("Stabileze") containing starch, sucrose, corn oil and silica [21]. Also, two bacterial bioherbicides (*Pseudomonas* spp.) were stabilized with this mixture that retained highly viable populations after one year [22]. Spores of the bioherbicide *C. truncatum*, formulated in a solid/perlite-cornmeal-agar mixture at 15˚C, exhibited an extended shelf-life beyond that of spores in liquid formulation, or in a solid/vermiculite mixture [23]. Other studies on *C. truncatum* showed that spores incorporated into wheat flour-kaolin granules ("pesta"), germinated at high levels (>85%) after storage for 1 year at 25˚C [24]. Other stu-

dies using pest a granules and *C. truncatum* microsclerotia demonstrated high viability of the organism after 10-year storage at 4˚C [25]. *C. truncatum* storage stability could also be improved by maintaining the pest a product at optimal water content or water activity [24] [26] [27] [28]. Survival of the bioherbicide *Sclerotinia minor* may be enhanced by altering storage container atmospheres and/or temperatures and water activity [29]. Various solid formulations of bioherbicides such as dusts, granules, pellets, wettable powders and encapsulated products have been discussed in relation to formulation and storage [30]. Cracked grains have been used successfully by a number of researchers [31] [32] [33] [34] as part of the formulation. Calcium alginate has been used to encapsulate fungal mycelium, spores, bacterial cells and other propagules of several bioherbicides that provided useful bioherbicide formulations with extended shelf-lives [35].

Freeze-drying (lyophilization) as a preservative method has been widely used in the food industry and has had some application to bioherbicides. For example, bacterial bioherbicides such as *Xanthomonas campestris* pv. *poae* and *Pseudomonas syringae* pv. *tagetis,* and mosaic viruses with bioherbicidal activity have been freeze-dried and stored for several years without loss of infectivity [8]. Other *Pseudomonas* species with weed-suppressive activity, and *Pseudomonas fluorescens* D7, a bioherbicide (D7®; Verdesian Life Sciences, LLC, Cary, NC) retained activity when prepared as freeze-dried preparations [36]. A freeze-dried preparation of a strain of *Myrothecium roridum* exhibited bioherbicidal activity on several weeds [37]. Conidia and mycelium of an isolate of *Fusarium tumidum* with bioherbicidal activity ongorse (*Ulex europaeus*) were preserved via freeze-drying [38], however, conidia of *Exserohilum longirostratum*, a bioherbicide for itchgrass (*Rottboellia cochinchinensis*), failed to germinate after freeze-drying [39].

The fungus *Myrothecium verrucaria* (Alb. and Schwein.) Ditmar:Fr. (strain IMI368023) (MV) has been shown to have bioherbicidal activity on several weeds [40] [41] [42]. Other studies in our laboratory showed MV had bioherbicidal activity against several weeds including: Kudzu (*Pueraria lobata* var. *montana*) [43], purslanes (*Portulaca* spp.) and spurges (*Euphorbia* spp.) [44], morninglory spp. (*Ipomoea* spp.) [45], hemp sesbania [46], and Palmer amaranth (*Amaranthus palmeri*) [47]. In some of these studies, we found synergistic interactions of the herbicide glyphosate and MV on weed control [46] [48] [49] [50]. Early research on MV utilized spores, but the production of trichothecenes by spores [51] limited its acceptance, usage and safety. However, this problem was circumvented by using MV mycelia preparations produced by liquid fermentation, thereby mitigating or vastly reducing the levels of these compounds, and the mycelial product was highly efficacious [52]. The liquid mycelial fermentation product exhibited some stability under refrigeration (4˚C - 5˚C), but storage of this heavy, liquid material proved to be bulky, cumbersome, and not conducive to providing an easy-to-use commercial product. The objectives of the

present experiments were to examine the effects of freeze drying on the efficacy and on long-term storage of MV mycelial preparations.

2. Materials and Methods

2.1. Isolation and Culture of *Myrothecium verrucaria*

A single strain of *M. verrucaria* (IMI 368023) was used throughout these studies. This strain was originally isolated from diseased sicklepod (*Senna obtusifolia*) [40]. The fungus was sub-cultured in Petri dishes containing potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA). Stock cultures were stored in twice-sterilized soil (25% water holding capacity) at 4˚C [20]. The inoculated plates were inverted and placed on open wire-mesh shelves of an incubator (Precision Scientific Inc., Chicago, IL, USA) at 28˚C. Photoperiods (12 h) were provided to induce sporulation [40] by 20 W cool-white fluorescent lamps to give ~200 $Em^2 \cdot s^{-1}$ photosynthetically active radiation (PAR) at dish level.

2.2. Production of *M. verrucaria* Mycelium

A soybean flour-cornflour medium (15.0 g soybean flour, 3.75 g cornflour, 30.0 g sucrose, 3.0 g calcium carbonate, per liter of distilled water) modified from [53], was used to produce high yields of mycelial cultures of *M. verrucaria* via fermentation in laboratory fermenters (Models MF-214 and CMF-128, New Brunswick Corp., Edison, NJ, USA) [52] (Boyette *et al.* 2008). Fermentations were conducted at: 185 - 200 rpm, 28˚C, 48 h. For these tests, the raw fermentation product (liquid, unspent medium, and mycelium) was homogenized in ~3 L aliquots using an electric blender (high speed, 3 min, Waring Model CB1043, Springfield, MO, USA). This freshly prepared mycelia material was used directly or freeze-dried and stored at −20˚C.

2.3. Lyophilization of MV Mycelial Product

Preliminary freeze-drying studies were carried out using a Virtis freeze dryer (Item 356923) (Los Angeles, CA, USA), and in later studies a Virtis, Freezemobile 25EL unit was utilized. Freshly fermented MV mycelium was blended (see above) to homogenize mycelial clumps, and then (~225 - 250 mL) was flash-frozen in shells (~10 - 15 mm thick) within the inside of the lyophilization flasks (600 mL) by rapidly rotating flasks in an ethanol bath at −60.9˚C. After the mycelium was completely frozen, the flasks were attached to the vacuum ports of the tree manifold of the freeze dryers and lyophilization was initiated. The frozen samples were freeze-dried at a condenser temperature of −80.8˚C and the samples were kept frozen during drying under a vacuum of 16milliTorr.After samples were dry, the mycelial powder was transferred to plastic bags, labeled and placed in storage (−20˚C) until further testing.

2.4. Hemp Sesbania Seedlings for Bioassay of MV Phytotoxicity

Two seedling bioassays (greenhouse-grown plants and hydroponically-grown

plants in the dark) were used in these experiments. For greenhouse-grown plants, scarified hemp sesbania seeds were surface sterilized (5% sodium hypochlorite; 10 min), rinsed with distilled water, and then planted in a commercial potting mix (Jiffy-mix, Jiffy Products of America, Inc., Batavia, IL) contained in peat strips (Jiffy Products). Each strip contained 12 plants and controlled-release (14:14:14, NPK) fertilizer (Osmocote, Grace Sierra Horticultural Products, Milpitas, CA) was incorporated in the soil mix. Growth occurred under greenhouse conditions [25˚C to 30˚C 40 to 90% relative humidity (RH), 12 h photoperiod (1,650 $Em^2 \cdot s^2$ photosynthetically active radiation)]. Seedlings in the late cotyledon growth stage were used for testing. Seedlings were treated via spray applications [hand-held sprayers (Crown Spra-Tool, North American Professional Products, Woodstock, IL, USA)] of freshly fermented or with freeze-dried mycelial preparations that had been stored at −20˚C for 8 years. The blended, freshly-fermented MV mycelium was deemed a 1.0x concentration and appropriate dilutions were made using sterilized H_2O. Freeze-dried MV samples were re-hydrated by slowly stirring the dried material in sufficient sterilized H_2O to bring product back to its original volume. In tests of spray applications to hemp sesbania seedlings, concentrations of 1.0x and 0.50x containing 0.10% Silwet L-77 (v:v) (non-ionic surfactant; OSi Specialties, Inc., Danbury, CT, USA) were used (dilutions made with deionized H_2O). Silwet at 0.10% in H_2O was used as a control treatment. Growth, disease symptomology and progression were monitored over a time course (15 days), and dry weight reduction measurements were determined 15 DAT. Disease progression on seedlings was monitored using a modified rating scale [54] of 0 to 10, where 0 = no injury and 10 = total necrosis (mortality). The second method was hydroponic growth under darkness wherein the hemp sesbania seeds planted in rolled paper toweling cylinders as described previously [55]. In this method, seed germination and growth was achieved in a high-humidity (100% RH) chamber under continuous darkness (80˚C). After 3-days growth under these conditions, uniform seedlings were selected and transferred to fresh paper toweling and grown an additional 24 h prior to treatment. In these young sensitive plants, MV samples was applied at a 0.25x rate for the hydroponic bioassay, and contained less Silwet, *i.e.*, (0.05%, v/v) than in greenhouse tests. Plants were spray-inoculated as described above, and then placed in a high humidity chamber (28˚C, 100% RH). Control plants received sprays of Silwet (0.05%) in water. This bioassay was conducted under the above conditions in the dark, for aduration of 96 h after spray inoculation. All experiments in both assay methods were arranged in randomized complete block designs, containing three replications and were repeated in time.

3. Results and Discussion

3.1. Efficacy Tests under Greenhouse Conditions

Freeze drying of the MV mycelium yielded a course, light brown colored powder (**Figure 1**). This material retained this color even after storage for 8 years at −20˚C.

Comparative greenhouse bioassay tests of fresh MV mycelium versus the 8-year old freeze-dried mycelium, using hemp sesbania seedlings as the target weed, showed only a 20% loss in activity (seedling dry weight reduction) when both samples were applied at the 1.0x rate (Figure 2). A greater loss in activity (~60%) of the freeze-dried material was demonstrated when seedlings were treated at the 0.50x rate.

Figure 1. Sample of freeze-dried *Myrothecium verrucaria* mycelial powder, stored 8 years at −20˚C.

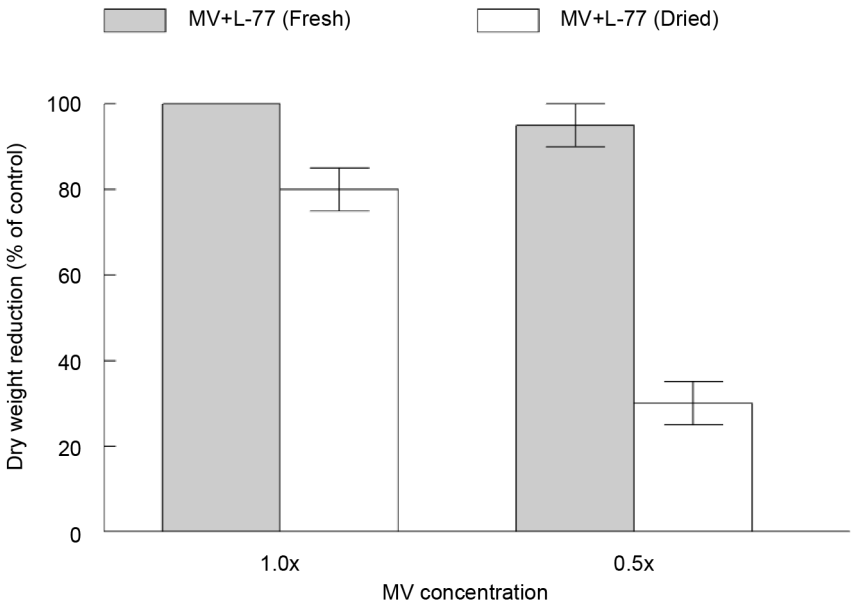

Figure 2. Mortality of hemp sesbania seedlings after treatment with freshly fermented *Myrothecium verrucaria* or freeze-fried mycelium preparations over a 15-day time course. Shaded histogram bars = freshly fermented *M. verrucaria* mycelium in 0.10% L-77 surfactant; open histogram bars = freeze-dried *M. verrucaria* mycelium, stored 8 years at −20˚C. Error bars represent ± 1 SE of means.

Examination of weed mortality in greenhouse-grown plants over a 15 day time-course showed that fresh MV caused ~85% mortality 3 days after treatment compared to a 30% mortality for the freeze-dried material (Figure 3).

The mortality values for fresh MV increased from 85% (day-3) to 100% by day-12, while the mortality caused by freeze-dried MV developed at a slower rate, achieving values of 65% by day-6 and 85% at day-15. Disease progression monitoring over the 15-day time course followed a trend similar to that for the mortality (Figure 4).

3.2. EfficacyTests under Dark Growth Conditions

Bioassays using hydroponically grown hemp sesbania seedlings under dark-growth conditions in an environmental chamber demonstrated relatively high bioherbicidal activity. In these tests, hypocotyl elongation was reduced 85%, 86% and 74% after treatment with freshly fermented MV, fresh freeze-dried MV (stored 5 months, −20˚C) and freeze-dried MV (stored 8 years, −20˚C), respectively (Table 1).

These growth bioassay tests are very sensitive since the plants are grown under continuous darkness and under hydroponic conditions. These results again demonstrated that bioherbicidal activity was retained by the freeze-drying process and by long-term storage of the freeze-dried MV mycelial material.

Overall, there was some activity loss of MV mycelium after 8 years of storage

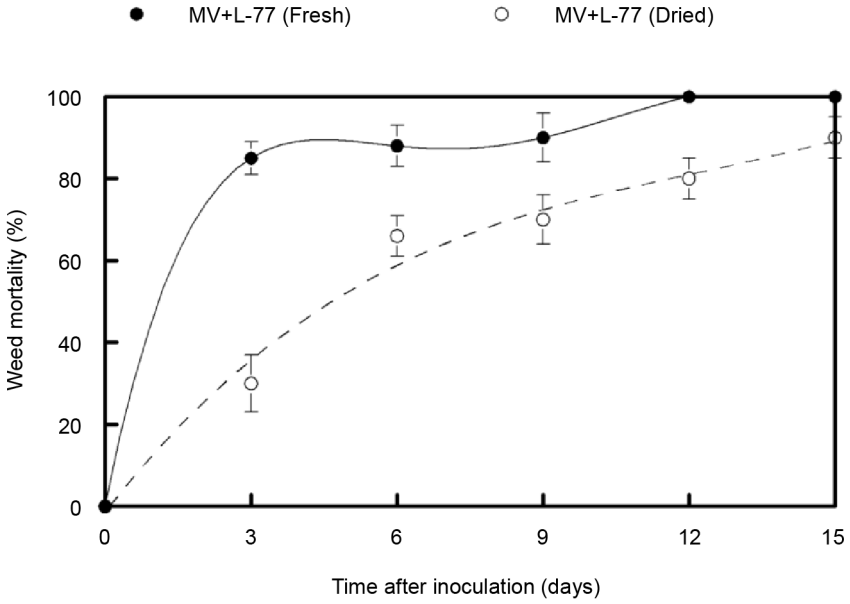

Figure 3. Mortality of hemp sesbania seedlings treated with freshly fermented *Myrothecium verrucaria* or freeze-fried mycelium preparations, 15 days after treatment. Shaded histogram bars = freshly fermented *M. verrucaria* mycelium plus 0.10% L-77 surfactant; open histogram bars = freeze-dried *M. verrucaria* mycelium that had been stored 8 years. Solid line = Fresh MV, $Y = -4.99 + 6.22\ X - 1.64X^2 + 0.20X^3$; $R^2 = 0.99$. Dashed line = Freeze dried MV, $Y = -0.02 + 1.44\ X - 0.09X^2 - 0.01X^3$; $R^2 = 0.97$. Error bars represent ± 1 SE of means.

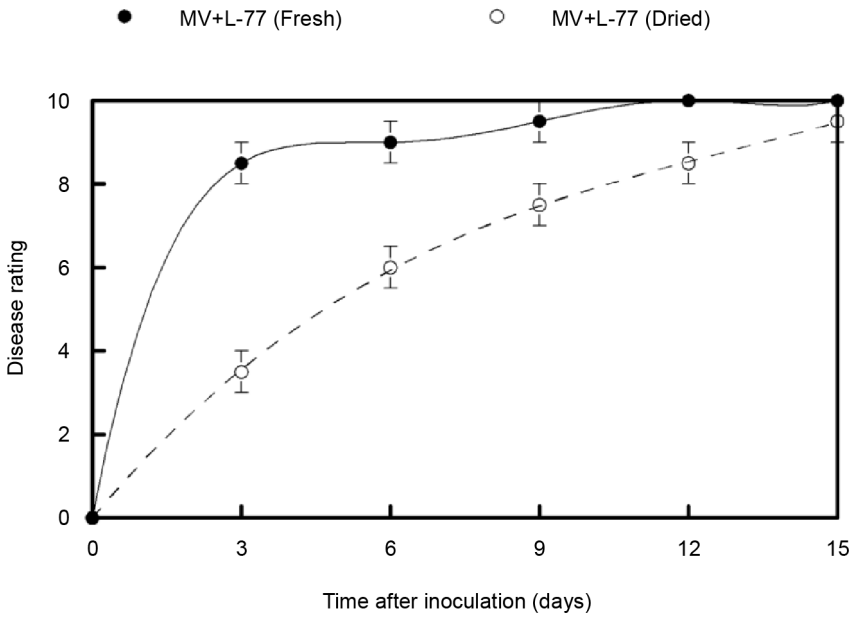

Figure 4. Disease progression after applications of freshly fermented *Myrothecium verrucaria* or freeze-fried mycelium preparations to hemp sesbania seedlings, over a 15-day time course. A modified rating scale (Horsfall and Barratt 1945) was used as described in Materials and Methods. Closed circles = freshly fermented *M. verrucaria* mycelium plus 0.10% L-77 surfactant; open circles = freeze-dried *M. verrucaria* mycelium that had been stored 8 years. Solid line = Fresh MV, $Y = 3.81 + 6.0\ X - 14.81X^2 + 1.65X^3$; $R^2 = 0.99$. Dashed line = Freeze dried MV, $Y = -1.62 + 15.38\ X - 1.05X^2 + 0.03X^3$; $R^2 = 0.98$. Error bars represent ± 1 SE of means.

Table 1. Effects of freshly fermented *Myrothecium verrucaria* mycelium, fresh freeze-dried MV mycelium and freeze-dried MV mycelium stored for 8 years at −20˚C on growth reduction (hypocotyl length) of hydroponically grown hemp sesbania seedlings, 96 hours after spray inoculation under dark conditions. Values presented are means followed by 1 S.E. in parenthesis.

Treatment	Hypocotyl length, mm (% of control)
Freshly fermented MV mycelium	15.1 (±1.1)
Freeze-dried MV mycelium (5-months old)	14.2 (±0.99)
Freeze-dried MV mycelium (8-years old)	25.8 (±1.3)

at −20˚C, however, the freeze-drying and freeze-storage technique appears to be a valuable method to preserve bioherbicidal activity of this material. Additionally, there may be other measures that could further improve storage stability of this freeze-dried bioherbicidal product. Perhaps storage at lower temperature (e.g., −80˚C) after freeze-drying would help. Also the freeze-dried product could be vacuum-sealed and/or stored frozen in an inert atmosphere to reduce or minimize oxidative processes. The preservation of various biological materials (including microorganisms) under desiccation has been shown to have utility [56] and could be examined with this bioherbicide. Thus, more research will be needed to address these possibilities. Future tests will also examine the efficacy

of this freeze-dried material more rigorously in greenhouse and field experiments on several weeds.

Even though freeze-drying has been shown to preserve MV mycelial preparations in these experiments, as well as in preservation of other fungal, bacterial and viral bioherbicides (as presented earlier), this method is not universal as demonstrated in unsuccessful attempts with spore preparations of a bioherbicde for itchgrass [39]. Perhaps the method has also failed with other bioherbicides, but not reported due to negative data. Therefore, each organism and/or formulation of an organism needs to be tested for stability under conditions associated with freeze-drying and long-term storage at −20˚C.

The overall stability of a living entity, such as a bioherbicide, to freeze-drying and freezing conditions is dependent on the nature of the biological material, as well as on possible interactions with formulation ingredients. In our experiments, MV mycelium was produced via fermentation on a soybean flour-corn flour mixture, supplemented with sucrose and calcium carbonate [53]. This biologically active MV product (used directly for weed control, or freeze-dried for stability tests) contained MV mycelium, unspent media components and possible MV compounds excreted into the medium. Sugars and polyhydroxy compounds that become more concentrated during freeze-drying or desiccation of microorganisms have been shown to play a role in stability through interactions with membranes, proteins and other biochemical constituents [56]. Therefore, such physicochemical interactions of the components of our fermentation media may have contributed positively to preserving the biological activity of this MV product.

4. Conclusion

To date, a large amount of information has been generated on the bioherbicidal potential of this MV strain. Generally, as pointed out above, it can affect and control many weeds, it acts synergistically with some herbicides (glyphosate) on certain weeds, it can control some weeds that are resistant to herbicides, the levels of toxic trichothecenes can be reduced or eliminated (via liquid culture fermentation), and now, a method to improve shelf life and extend long-term storage has been demonstrated. All of these factors are important in improving the bioherbicidal potential of this agent.

Acknowledgements

The authors thank Robin H. Jordan for expert laboratory and data graphics assistance during this project.

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