

Solid Substrate Formulations of the Mycoherbicide *Colletotrichum truncatum* for Hemp Sesbania (*Sesbania exaltata*) Control

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Colletotrichum truncatum was grown on kernels of eight different grains for 3 or 4 weeks at room temperature (22–24°C). Fresh preparations of conidia as well as fungus-infested corn and rice suspensions resulted in 100% mortality of hemp sesbania seedlings when sprayed post-emergence with a 14 h dew period. Fresh preparations of mycelia and fungus-infested sorghum suspensions resulted in 90 and 65% mortality of hemp sesbania seedlings, respectively. Lower mortality ($\leq 15\%$) occurred with the other ground fungus-infested grain suspensions. Fresh preparations of conidia, fungus-infested corn, rice and sorghum, and mycelia, when applied to soil pre-emergence, resulted in 100, 94, 100, 83 and 71% mortality of hemp sesbania seedlings 14 days after application, respectively. Lower mortality ($\leq 23\%$) occurred with the other ground fungus-infested grain preparations. Freshly-prepared *C. truncatum* at 6.25, 12.5, 25 and 50 mg fungus-formulated rice cm^{-2} of soil surface, applied pre-emergence or at the time of planting, killed 97, 100, 100 and 100% of hemp sesbania, respectively. After storage at 22–24°C for 6 to 24 months, the rice formulation caused 67 to 93% mortality after 6 months, 39 to 81% after 12 months, and $\leq 2\%$ after 24 months, respectively. When *C. truncatum* was refrigerated at 4–6°C, the rice formulation retained good efficacy through 24 months, and when frozen, for up to 8 years. *C. truncatum* formulated on rice stored under all the above conditions contained mainly sclerotia at 2.4×10^5 sclerotia g^{-1} . *C. truncatum* killed hemp sesbania seedlings with a single soil application through 4 plantings on the same soil. These results indicate that rice and possibly corn are excellent solid substrates for the formulation of *C. truncatum*. This is a simple and effective method for enhancing the activity of *C. truncatum* against hemp sesbania.

Keywords: formulation, solid formulation, biological control agent, mycoherbicide, grains, shelf-life, *Colletotrichum truncatum*, *Oryza sativa*

INTRODUCTION

Hemp sesbania (*Sesbania exaltata* [Rydb.] ex A. W. Hill) is a serious weed problem in the southern USA, affecting soybean [*Glycine max* (L.) Merr.] (McWhorter & Anderson, 1979;

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Jordan, 1985), cotton (*Gossypium hirsutum* L.) (Bryson, 1987) and rice (*Oryza sativa* L.) (Smith, 1968; Khodayari & Smith, 1988). *Colletotrichum truncatum* (Schw.) Andrews and Moore is a highly virulent, specific pathogen of hemp sesbania, but is limited in its usefulness by a short shelf-life, extended dew period requirement and limited host range (Boyette, 1991a,b; Boyette *et al.*, 1993b). These problems have been overcome with other pathogens by the use of formulation and application techniques (Connick *et al.*, 1993; Boyette *et al.*, 1993a; Boyette & Abbas, 1994, 1995; Abbas & Egle, 1996; Daigle *et al.*, 1998).

Silman *et al.*, in 1991 studied membranes, liquid culture and solid particles such as vermiculite and rice hulls for production of *C. truncatum*. These methods produced mainly conidia. Furthermore, Silman and Nelsen (1993) and Jackson and Schisler (1992) studied optimization of liquid culture medium, producing conidia. Drying and application methods were not investigated. In addition, *C. truncatum* formulated in a wheat gluten matrix has potential for controlling hemp sesbania (Connick *et al.*, 1993, 1997).

We have successfully produced fungi on grains, particularly rice and corn, enabling the growth of fungi under controlled conditions and allowing preservation of fungal plant pathogens, such as *Fusarium* spp. and *Alternaria* spp., for years at temperatures ranging from 4 to -20°C (Abbas *et al.*, 1991, 1993).

C. truncatum can exist as conidia, mycelia and in a dormant phase, sclerotia. Growth on small grains produces mainly sclerotia. Sclerotia survive the winter and are more resistant to extreme environmental conditions.

Mycoherbicide products applied to the soil before or during planting, or to the emergent plants, can provide excellent weed control (Boyette *et al.*, 1984; Boyette & Walker, 1986). This study examines the production and use of *C. truncatum* in grain-based solid substrates to control hemp sesbania and which could make this product more useful commercially.

MATERIALS AND METHODS

Fungus and Growth Conditions

The culture of *C. truncatum* used in all tests was isolated from infected hemp sesbania plants in the weed nursery in Stoneville, MS, in 1987, and has been deposited in the ARS Culture Collection (NRRL) at the National Center for Agricultural Utilization Research, Peoria, IL, USA (NRRL#18434). Inoculum (conidia and mycelium) for all experiments was produced in Petri dishes containing Difco potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA). Inoculated Petri plates were incubated in an incubator (Precision Scientific Inc., Chicago, IL 60547, USA) at $26\text{--}28^{\circ}\text{C}$ with a 12 h photoperiod under fluorescent light for 8–10 days (Boyette, 1991a,b; Templeton, 1992).

Inoculum Production

For mycelium production, 1–2 cm^2 pieces from PDA plates carrying mycelium and conidia were inoculated into flasks of autoclaved medium consisting of cornmeal (0.44 g), soy flour (3.75 g), sucrose (7.5 g) and calcium carbonate (0.75 g) in 600 ml of deionized water. The inoculated flasks were shaken at 165 rpm for 10 days at $26\text{--}28^{\circ}\text{C}$ (G24 Environmental Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA) when the fungal biomass culture was filtered onto filter paper and then air dried for 3 to 5 days prior to use in mycelium formulations (Walker & Riley, 1982).

Conidial inoculum was prepared by spreading 1 ml of *C. truncatum* conidial suspension (1×10^7 conidia ml^{-1}) onto PDA plates. Plates were inverted and incubated under fluorescent light at $26\text{--}28^{\circ}\text{C}$ for 8 to 10 days. The resulting conidia were rinsed from plates with sterile distilled water, counted in a hemacytometer and adjusted to desired concentrations by adding distilled water.

Preparation of Small Grains Infested With Fungus

Barley (*Hordeum vulgare* L.), corn (*Zea mays* L.), cotton, oats (*Avena sativa* L.), rice, sorghum [*Sorghum bicolor* (L.) Moench], soybean, and wheat (*Triticum aestivum* L.) obtained

TABLE 1. The average numbers of sclerotia formed on grains infested with *Colletotrichum truncatum*

Type of inocula	Rate of sclerotia formation (Sclerotia g ⁻¹) ^a
Potato-dextrose agar (PDA)	2.4 × 10 ³ d
Rice-formulation	2.4 × 10 ⁵ a
Corn-formulation	2.1 × 10 ⁵ a
Sorghum-formulation	1.5 × 10 ⁵ b
Other grain-formulations ^b	≤ 3.5 × 10 ⁴ c

^aRate is a mean of three determinations. Values in columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

^bOther grains included barley, cotton, oats, soybeans, and wheat.

from Cargill Corp., Greenville, MS, USA, were used as substrates for *C. truncatum*. *C. truncatum* was grown on small grains as described by Abbas and Boyette (1991) and Abbas *et al.* (1991). Briefly, 1 l flasks containing 200 g of grains and 120 ml distilled water were autoclaved twice. The media was inoculated with a 1–2 cm² PDA plug containing conidia and mycelia of *C. truncatum*. The flasks were placed on laboratory benches for 2–3 weeks at room temperature (22–24°C) under fluorescent light. Fungal colonization on corn, rice, and sorghum reached 100% by visual inspection under these conditions, but colonization of the other grains was visibly less (Abbas & Boyette, 1991). The fungus-infested grains were harvested, air dried under a ventilated hood for 3–5 days and ground to the consistency of flour, about 2 mm. The fungus formed mainly sclerotia, which are more resistant to environmental conditions. Sclerotia numbers were recorded for each grain (Table 1). Fungal colonization was measured in two separate experiments, each consisting of three replications containing 100 kernels of grain each.

Biological Testing

From each of the eight fungus-infested grains 5 g was homogenized in 50 ml autoclaved distilled water for 3–5 min in a Brinkmann Polytron^R Homogenizer, PT 3000 (Brinkmann Instruments Inc., Westbury, NY 11590-9974, USA), at 27.3×10^3 rpm. The homogenate was drained through double layers of cheese cloth to remove coarse particles and sclerotia were counted with a hemacytometer. Suspensions of conidia (1×10^7 ml⁻¹) or mycelium (2 g dry weight 50 ml⁻¹) in autoclaved distilled water were also prepared. All these preparations were sprayed on 7–10 day old (first true leaves) hemp sesbania seedlings until runoff occurred. Sixty hemp sesbania seeds were planted in each 5 cm pot with 50% Jiffy mix (a commercial potting mixture supplemented with a slow release N:P:K, fertilizer (14:14:14), purchased from Jiffy Products of America, Inc., Batavia, IL, USA) in local sandy loam soil. Pots were thinned to 20 seedlings each. Treated plants were given a 14 h dew period by putting them in dew chamber (Percival Scientific, Inc., Boone, Iowa 50036, USA) and then placed in the greenhouse at 28 to 32°C with 40 to 60% relative humidity (RH). The photoperiod was approximately 14 h, with 1600 to 1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) at midday, as measured with a light meter for 14 days. Treated and nontreated plants were observed daily and morphological changes and mortality recorded. Control plants were sprayed with autoclaved distilled water or homogenate of 5 g of autoclaved rice with no fungus.

In a pre-emergence application, the treatments were applied by sprinkling directly on the soil after sowing 60 hemp sesbania seeds per pot and not subjected to a dew period. Fungus-infested grains, conidia, and mycelium were applied at rates of 50 mg cm⁻² of soil surface, 4 cm pot⁻¹ at 1×10^7 conidia ml⁻¹, 50 mg cm⁻² of soil surface, respectively. Means were

calculated from two separate experiments, each consisting of three replications containing 20 plants per replication.

Dose-Response of Hemp *Sesbania* to Fungus

Only the rice and corn formulation of the fungus were compared with conidia and mycelium. The inocula were prepared as described above and applied immediately to the soil surface at the time of sowing (pre-emergence). Conidia were applied at 1.3×10^4 , 2.5×10^5 , 5×10^6 and 1×10^7 conidia ml^{-1} while fungal mycelium, and rice and corn formulations of the fungus were applied by aerosol sprayer until run-off at rates of 6.25, 12.5, 25, and 50 mg cm^{-1} of soil surface in 5 cm pots. Three replications were made, each containing 60 seeds (thinned to 20 plants) and the experiment was repeated twice. Treated and control plants were kept in the greenhouse. Plant mortality was evaluated 14 days after treatment.

Shelf Life of *C. truncatum*

Only the fungus-formulated rice and corn were used to study shelf life compared with fungal conidia and mycelium. The inocula, prepared as described above, were sprayed until runoff as soon as prepared (1 day), and after 30 and 60 days storage at 4–6°C, onto first true leaf stage hemp sesbania seedlings.

Treated and untreated plants were subjected to 14 h dew periods prior to being kept in the greenhouse. Conidia were sprayed at a rate of 1×10^7 conidia ml^{-1} , fungal mycelium at 2.5 g mycelium 50 ml^{-1} water, and rice and corn formulations at 5 g infested grain 50 ml^{-1} water, for conversion to sclerotia g^{-1} , see Table 1. All inocula were stored at 4–6°C as dry powder, except conidia which were stored in suspension, for 30 and 60 days. Three replications were used, each containing 20 plants, and the experiment was repeated twice. Plant mortality was evaluated 14 days after treatment.

As the rice-formulation caused high mortality of hemp sesbania seedlings, it was chosen to study prolonged shelf life. One 2 kg portion of fungus-infested rice was stored at -20°C for various times, another portion was stored at 4°C and a third portion was stored at room temperature, 22–24°C. All these were tested for virulence against hemp sesbania seedlings 0, 6, 12 and 24 months storage compared to freshly-prepared rice inoculum. In addition, inoculum stored at -20°C was tested against hemp sesbania after storage for 6, 7 and 8 years. Five rates of inoculum 0, 6.25, 12.5, 25 and 50 mg cm^{-2} of soil surface (which corresponded to 0, 1.5×10^3 , 3×10^3 , 6×10^3 and 1.2×10^4 sclerotia cm^{-2} , respectively) were applied with the seeds at the time of sowing (pre-emergence). Thirty seeds were used for each pot. Each treatment was in triplicate and the experiments were repeated twice. Seedling mortality was evaluated 2 weeks after planting.

Persistence of *C. truncatum* in Soil

Freshly prepared fungus-infested rice was applied (pre-emergence) at 6, 12.5, 25 and 50 mg grain cm^{-2} of soil surface when hemp sesbania seeds (60 seeds per 5 cm pot) were planted. Three replicates for each treatment were used, including two control groups consisting of untreated plants and plants treated with ground autoclaved rice at the same rates as the infested rice. All plants were kept in the greenhouse for 14 days. After two weeks, the same pots were replanted and mortality was evaluated after a second 2-week period. This procedure was repeated five times. Experiments were performed twice in two different laboratories.

RESULTS

Ten days after inoculation, *C. truncatum* had colonized corn and rice grains at 55 and 65%, respectively. *C. truncatum* colonized sorghum grains at 25% within 10 days, while the

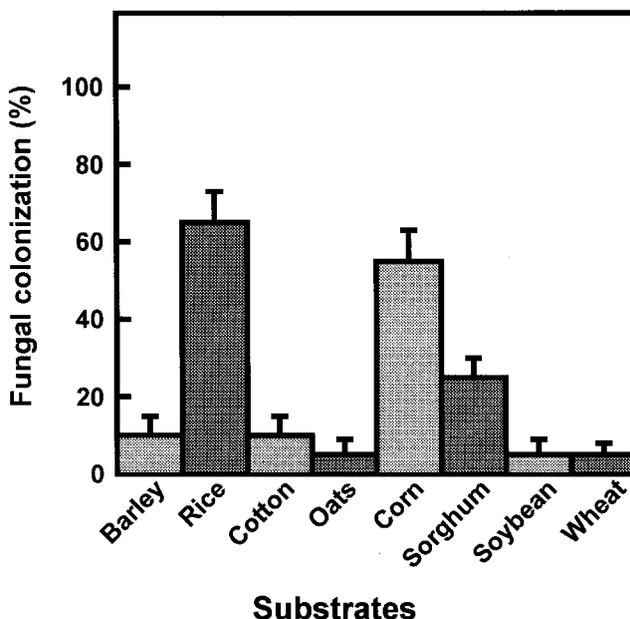


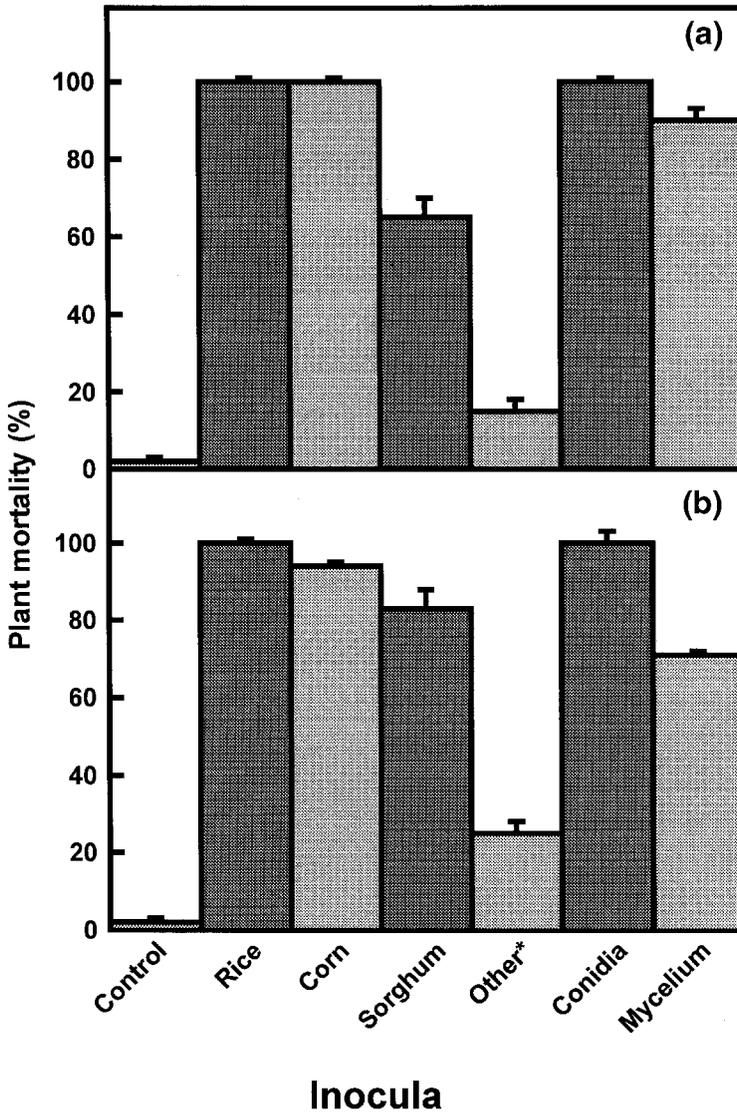
FIGURE 1. Colonization of *C. truncatum* on various grains after 10 days. Note fungal colonization was most extensive on rice (65%) and corn (55%) kernels. Vertical bars represent 95% confidence intervals from two separate experiments, each consisting of three replications containing 100 kernels of grain each.

remaining grains were colonized by 5 to 10% 10 days after inoculation (Figure 1). After 3 weeks, fungal colonization on rice and corn had reached 100% and sorghum 85%, but the fungal colonization of the other grains was visibly less. Soybean and cotton seeds did not promote growth of *C. truncatum*. *C. truncatum*-infested grains did not produce significant conidia, but mainly sclerotia in various numbers depending on the substrates (Table 1).

When the conidia, mycelium, and the fungus-infested rice and corn suspensions were applied to seedlings immediately after preparation, 90–100% mortality occurred within 14 days. The fungus-infested sorghum suspension caused 65% mortality. The other grain suspensions were less effective, resulting in mortality between 5 and 15% (Figure 2(a)). When the same inocula were applied pre-emergence to hemp sesbania seeds at the soil surfaces, 94–100% mortality resulted within 14 days following treatment with the fungus-infested corn and rice suspensions, respectively. Fungus-infested sorghum suspension killed 83% of hemp sesbania during this time. The remaining fungus-infested grains (oats, barley and wheat) caused 23, 21 and 23% mortality, respectively. The mycelia and conidia suspensions resulted in 71 and 100% mortality, respectively (Figure 2(b)).

In the study of dose response, conidia caused 100% hemp sesbania mortality at rates of 1.3×10^4 , 2.5×10^5 , 5×10^6 and 1×10^7 conidia ml^{-1} when applied to soil surfaces. Mycelium inoculum when applied at rates of 6.25, 12.5, 25 and 50 mg cm^{-2} of soil surface, caused 42, 67, 68 and 73% mortality on hemp sesbania. Fungus-infested rice caused 65, 85, 88 and 100% mortality and fungus-infested corn caused 67, 79, 90 and 96% mortality when applied to the soil surface at 6, 12.5, 25 and 50 mg cm^{-2} (Table 2). In all cases, higher concentrations of fungal inoculum caused higher mortality of hemp sesbania.

Freshly prepared conidia at $1 \times 10^7 \text{ ml}^{-1}$, sprayed to run-off, caused 100% mortality to hemp sesbania (Table 3). However, after 30 days storage at 4–6°C, conidia caused no mortality. This is consistent with the findings of Jackson *et al.* (1995). Fresh fungal mycelial



*Others = Barley, cotton, soybean, oats & wheat

FIGURE 2. (a) Mortality of first true leaf stage hemp sesbania (*Sesbania exaltata*) when sprayed with various grain formulation of fungus, conidia and mycelium and assessed 14 days after initial application. Inoculum concentrations were 5 g 50 ml⁻¹ formulated fungus, 1 × 10⁷ conidia ml⁻¹, and 2 g mycelium 50 ml⁻¹. Means were from two separate experiments, each consisting of three replications containing 20 plants per replication. (b) Mortality of hemp sesbania (*S. exaltata*) 14 days after treatment with grain-formulation of fungus, conidia, and mycelium. Only the conidia were suspended in water before application. The inocula were applied to the soil at time of planting of seeds (pre-emergence) at 50 mg cm⁻² grain formulated fungus, 1 × 10⁷ conidia ml⁻¹, and 50 mg cm⁻² mycelia. Means are from two separate experiments, each consisting of three replications containing 60 seeds per replication. Vertical bars represent 95% confidence intervals.

TABLE 2. Dose responses of various inocula of *C. truncatum* on hemp sesbania growth when applied at the time of planting (pre-emergence)

Type of inocula	Unit used and mortality ^a	Inoculum dose			
		Rate 1	Rate 2	Rate 3	Rate 4
Conidia	Conidia ml ⁻¹	1.3 × 10 ⁴	2.5 × 10 ⁵	5 × 10 ⁶	1 × 10 ⁷
	Mortality	100% a	100% a	100% a	100% a
Mycelia	mg cm ⁻²	6	12.5	25	50
	Mortality	42% d	67% c	68% c	73% c
Rice-formulation	mg cm ⁻²	6	12.5	25	50
	Mortality	65% c	85% b	88% b	100% a
Corn-formulation	mg cm ⁻²	6	12.5	25	50
	Mortality	67% c	79% b,c	90% b	96% a

^aMeans were from two separate experiments, each consisting of three replications containing 60 seeds/replication. Values in columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

TABLE 3. Shelf life of different formulations of *C. truncatum* stored at 4–6°C measured as their effect on hemp sesbania mortality after post-emergence application

Type	Inocula		Mortality (%) of hemp sesbania after days storage ^a		
	Form stored	Rate sprayed	1	30	60
Conidia	Suspension	1 × 10 ⁷ ml ⁻¹	100 a	0 d	NT
Mycelium	Powder	2.5 g 50 ml ⁻¹	100 a	95 ± 7.5 a,b	75 ± 1.2 c
Rice-formulation	Powder	5 g 50 ml ⁻¹	100 a	100 a	100 a
Corn-formulation	Powder	5 g 50 ml ⁻¹	100 a	98 ± 5.3 a	93 ± 6.6 b

^aMeans were from two separate experiments, each consisting of three replications containing 20 plants per replication. Values in columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

inoculum caused 100% mortality to hemp sesbania when applied at a rate of 2.5 g 50 ml⁻¹ but, again, only 95 and 75% mortality, respectively, after 30 and 60 days storage at 4–6°C (Table 3). Fungus-infested corn and rice retained high activity after 60 days storage, causing 93–100% mortality, respectively but there was a trend to decreasing efficacy with time for the corn formulation. Fungus-infested rice remained active (76% mortality) after 0.5, 1 and 2 years storage at 4–6°C (Figure 3). The same inoculum remained active during 6 months storage at room temperature (22–24°C), producing 67–93% mortality when applied at rates of 6–50 mg cm⁻². However, this activity decreased after 1 year, with mortality reduced to 39–81% (Figure 3). The inoculum had completely lost activity after 24 months at room temperature (Figure 3). The rice formulation stored frozen (–20°C) for 6–96 months remained viable causing 80–100% mortality of hemp sesbania seedlings (Figure 3). Inoculum of *C. truncatum* on rice remained viable in soil through four successive plantings of hemp sesbania, a total time of about 10 weeks (Figure 3). The efficacy remained high for the first two plantings but then, with the three lower dose rates, decreased to produce only about 40% mortality. At the higher dose rate, the decrease occurred after the third planting.

DISCUSSION

Among the solid substrates tested, rice and corn promoted optimum growth of *C. truncatum* resulting in high mortality of hemp sesbania seedlings. They also retained their activity much longer when refrigerated or frozen. Even when stored at room temperature they remained viable for up to 6 months. *C. truncatum* grown on solid substrates mainly exists in the form of sclerotia. Sclerotia are a more resistant structure to environmental conditions.

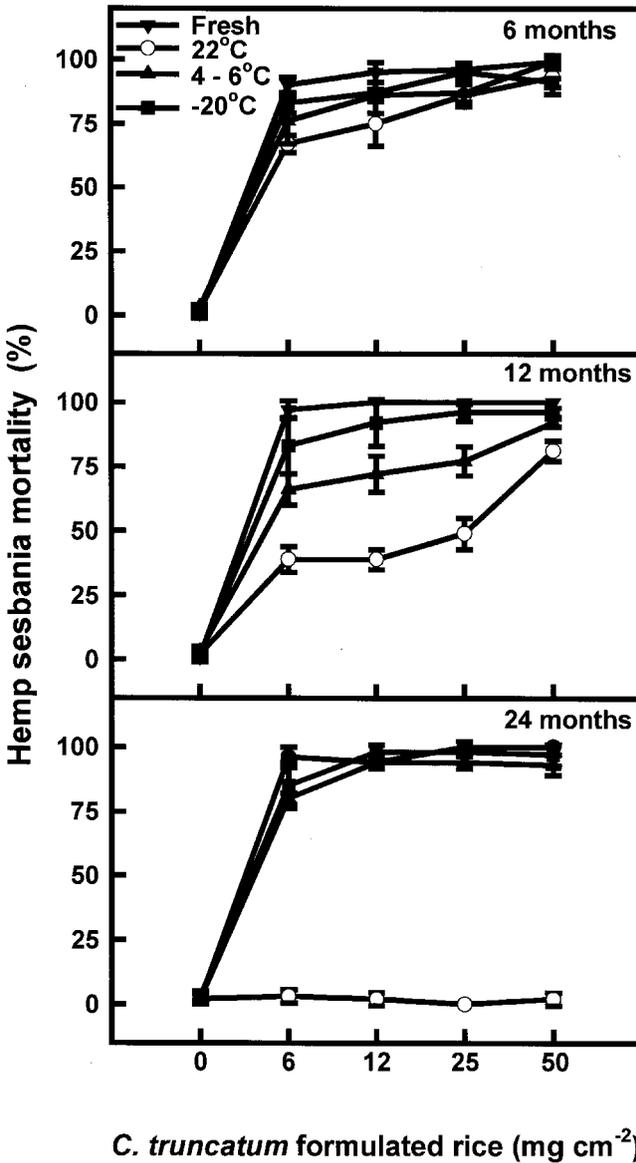


FIGURE 3. Effect of grain-formulated fungus on hemp sesbania mortality after 14 days. Inocula were stored at (22–24°C, 4–6°C and –20°C) for 6, 12 and 24 months and applied to soil at 0, 6.25, 12.5, 25 and 50 mg cm⁻² (containing 2.4×10^5 sclerotia g⁻¹ at time of seed sowing) (pre-emergence). Means were from two separate experiments, each consisting of three replications containing 60 seeds. Vertical bars represent 95% confidence intervals.

When microsclerotia of *C. truncatum* are incorporated into soil, they germinate sporogenically, the conidia produced causing infection of hemp sesbania (Schisler & Jackson, 1996). It is not known whether these solid substrates stimulate conidia formation from microsclerotia.

Although *C. truncatum* is an effective pathogen for the control of hemp sesbania, its usefulness has been limited by the need to use the material immediately after production

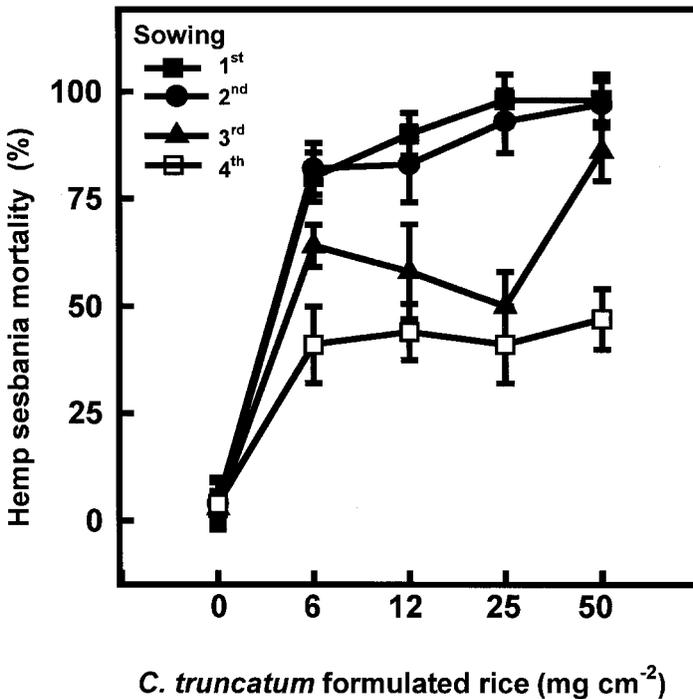


FIGURE 4. Persistence of rice-formulated fungus in soil measured as plant mortality after the inocula were applied to soil at the time of initial sowing (pre-emergence) at rates of 0, 6.25, 12.5, 25 and 50 mg cm⁻². Sowings were made at 14 day intervals. Means were from two separate experiments, each consisting of three replications containing 60 seeds each. Vertical bars represent 95% confidence intervals.

and only under specific environmental conditions. This study has demonstrated that it is possible to increase the shelf life of *C. truncatum* for significant periods by formulation in solid rice or corn substrates. At room temperature, shelf life extends across a planting season but can be extended greatly when refrigerated or frozen. The current study has demonstrated efficacy for up to 8 years. Research continues with this formulation in the field. This will produce more information on inoculum dosages. Preliminary results look promising. The practicality of this approach also needs to be investigated, particularly the large volume of inoculum currently needed. Production of a reliable, inexpensive solid substrate formulation will improve the bioherbicidal potential of this fungus.

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