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Chlamydospores of *Fusarium oxysporum* Schlecht f.sp. *orthoceras* (Appel & Wollenw.) Bilai as inoculum for wheat-flour–kaolin granules to be used for the biological control of *Orobanche cumana* Wallr.

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Abstract

The formation of chlamydospores of *Fusarium oxysporum* Schlecht. f.sp. *orthoceras* (Appel & Wollenw.) Bilai, a potential mycoherbicide for the parasitic weed *Orobanche cumana* Wallr., was optimised regarding the composition of the liquid growth medium. After 16 days of incubation, highest chlamydospore counts (approximately 2×10^7 ml^{-1}) were determined in an aqueous solution amended with 1.5% (w/v) finely ground sorghum stover and 30% (v/v) wheat-based stillage, a liquid by-product of ethanol production. The produced fungal biomass retained viability after being air-dried and stored. Granules made from wheat-flour and kaolin (Pesta) containing microconidia or air-dried chlamydospore-rich biomass were prepared and showed high efficacy in controlling *O. cumana* in the greenhouse. No differences in efficacy were observed between the different types of inoculum used.

Introduction

Root-parasitic weeds of the genus *Orobanche* (Orobanchaceae) are a serious threat to a wide range of economically important crops in warm-temperate as well as subtropical and tropical regions (Parker and Riches, 1993). *Orobanche cumana* Wallr. (sunflower broomrape) has become a limiting factor to sunflower (*Helianthus annuus* L.) production in Spain, the Balkan region, Turkey, and countries of the former USSR (Parker, 1994). It has also been introduced to Israel (Joel, 1988) and Eastern Asia (Liu and Li, 1988).

No control measure, which is effective and economically feasible at the same time, has been found for *Orobanche* spp. In the last years, the integration of biological methods into the control concepts was of increasing research interest. *Fusarium oxysporum* Schlecht. f.sp. *orthoceras* (Appel & Wollenw.) Bilai was isolated in Bulgaria from diseased *O. cumana* shoots and identified as a pathogen of the parasite which eventually kills the plant (Bedi and Donchev, 1991). Its host-range was apparently restricted to *O. cumana* and the closely related *O. cernua* (Thomas et al., 1998). In small-scale field trials in Bulgaria, Bedi (1994) achieved successful control of the parasite using the fungus grown on barley grains or a wheat straw/maize feed mixture as pre-planting applied inoculum. The efficacy of the soil application was tracked down to the susceptibility of early developmental underground stages of *O. cumana* to the pathogen (Thomas et al., 1999).

Further success of *F. oxysporum* f.sp *orthoceras* in agricultural applications will depend on the development of an appropriate formulation which allows storage, handling, and a successful soil-application of the fungus. Connick et al. (1991) developed a method to produce wheat-flour–kaolin granules (Pesta) by mixing fungal inoculum with wheat-based substrates,

a filler, and water. The readily available nutrient source permits rapid proliferation of the propagules under appropriate environmental conditions. Microconidia of *F. oxysporum* matrix-encapsulated in 'Pesta' granules were used by Boyette et al. (1993) for biological control of sicklepod (*Senna obtusifolia*), coffee senna (*Cassia occidentalis*), and hemp sesbania (*Sesbania exaltata*) under greenhouse conditions.

Chlamydospores formed by *F. oxysporum* play an important role in long-term survival of the fungus due to their resistance to desiccation and temperature extremes (Nash et al., 1961; Schippers and Van Eck, 1981) which makes them suitable propagules for a dry mycoherbicidal formulation. However, production of chlamydospores of *F. oxysporum* in mass quantities was time-consuming (Hildebrand and McCain, 1978) until Hebbar et al. (1996; 1997) developed a method for producing chlamydospore-rich biomass of mycoherbicidal strains of *F. oxysporum* in a one-step liquid fermentation process using water extract of soya bean (*Glycine max*) hull fibre as substrate.

The objectives of this study were to find a method for the mass-production of chlamydospores of *F. oxysporum* f.sp. *orthoceras*, to determine if the formulation process for wheat-flour–kaolin granules could be used with this mycoherbicidal agent, and to test the capability of the formulated material to control *O. cumana* in greenhouse trials.

Materials and methods

Fungal isolate

An isolate of *F. oxysporum* f.sp. *orthoceras* was received from Dr. J.S. Bedi (Punjab Agricultural University, Ludhiana, India) in 1995 (Thomas et al., 1998). Stock cultures were maintained on Special Nutrient-poor Agar (SNA, Nirenberg 1976). For long-term storage, the isolate was cryopreserved on SNA amended with 5% (v/v) glycerol.

Production of chlamydospores by F. oxysporum *f.sp.* orthoceras

Effect of different substrates on chlamydospore formation by F. oxysporum *f.sp.* orthoceras

In the first experiment, 1.5% (w/v) of very finely milled (<500 μm) sorghum stover (dried stalks) was tested together with different additives: (a) sorghum stover in deionised H_2O; (b) sorghum stover in a salt solution (1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 2 mg glucose per litre H_2O); (c) sorghum stover in a 2% celery extract solution (prepared by autoclaving 20 g of celery in 100 ml H_2O and filtering the extract through a filter paper); (d) sorghum stover with 1 g yeast extract per litre H_2O; (e) sorghum stover with 10 ml of 'thin' rye-based stillage (the spent fermentation broth of ethanol production, kindly provided by the Institute for Food Technology, University of Hohenheim), using only the supernatant, in H_2O, and (f) sorghum stover with 10 ml of 'thick' rye-based stillage, using the entire liquid, in H_2O.

Erlenmeyer flasks (250 ml, three replicates) containing 100 ml of the investigated media were autoclaved for 20 min at a temperature of 121 °C and aseptically inoculated after cooling with one agar plug (0.6 cm dia.) from an actively-growing culture of *F. oxysporum* f.sp. *orthoceras* on SNA. The cotton-plugged flasks were incubated on a rotary shaker at 100 rpm and room temperature for a period of 16 days. The liquid medium with the fungus was blended in a Waring blender and a small portion was passed through a tissue grinder. The numbers of microconidia and single-celled, thick-walled, and rounded chlamydospores were determined with a hemacytometer.

Effect of different straw and stillage types and their concentrations on conidiation and chlamydospore formation of F. oxysporum *f.sp.* orthoceras

The parameters evaluated were (a) the effect of four different stillage types (potato, triticale, wheat, and maize) at a rate of 20% (v/v) together with 1.5% (w/v) sorghum stover; (b) the effect of three finely milled and sieved (<500 μm) straw types (sorghum, maize, or wheat) at a rate of 1.5% (w/v) together with 20% (v/v) wheat-based stillage; (c) the effect of different concentrations (20%, 30%, 40%, and 50% (v/v)) of wheat-based stillage together with 1.5% (w/v) sorghum stover, and (d) the effect of different concentrations (0%, 0.5%, 1%, and 1.5% (w/v)) of sorghum stover together with 20% (v/v) wheat-based stillage on the formation of chlamydospores by *F. oxysporum* f.sp. *orthoceras*. Inoculation, culturing, and chlamydospore counting were conducted as described above.

Effect of plant fibres on chlamydospore formation by F. oxysporum *f.sp.* orthoceras

The effect of plant fibres on the chlamydospore formation by *F. oxysporum* f.sp. *orthoceras* was

investigated by growing the fungus in unfiltered and filtered liquid media, respectively (Hebbar et al., 1996). A medium containing 1.5% (w/v) sorghum stover and 20% (v/v) wheat-based stillage in deionised H_2O was autoclaved, and half of it was filtered through filter paper (Schleicher & Schuell No. 595). Erlenmeyer flasks (250 ml, three replicates) were filled with 100 ml of filtered and unfiltered medium, respectively, and re-autoclaved. Inoculation, culturing, and chlamydospore counting were conducted as described above.

Air-drying of biomass

Since no effective technique was found to separate the fungal propagules from the fibrous material, the substrate had to be part of the end-product. After a fermentation process in 1.5% sorghum stover and 30% (v/v) wheat-based stillage, biomass was gained by filtration through a cheesecloth. Colony Forming Units (CFUs) per gram of fresh weight were determined by plating serial dilutions on PDA plates amended with 200 ppm chloramphenicol and 100 ppm streptomycin sulphate (three plates per each of three samples) and corrected for sample moisture content. The biomass was air-dried and CFUs g^{-1} were determined again. The inoculum was stored in plastic bags at room temperature in the dark and CFUs g^{-1} were determined after 10 months of storage.

Encapsulation of F. oxysporum *f.sp.* orthoceras *into wheat-flour–kaolin granules*

Inoculum production

Fungal biomass abundant in chlamydospores was obtained using the liquid fermentation in an aqueous sorghum stover/wheat stillage medium described above. Microconidia were grown in liquid culture using 250-ml Erlenmeyer flasks containing 100 ml autoclaved potato dextrose broth (PDB, Sigma, Germany). One agar plug (0.6 cm dia.) from a fungal culture on SNA was used to inoculate each flask. The flasks were closed with a cotton plug and aluminium foil and incubated on a rotary shaker (125 rpm) at ambient laboratory conditions (±20 °C) for five days. The content of the flasks was homogenised for 30 s in a blender and mycelial fragments were separated by filtration through a four-layer cheesecloth. If necessary, CFU ml^{-1} were determined by plating 100-µl aliquots on half-strength PDA plates that were incubated for three days at room temperature.

Preparation and shelf-life of wheat-flour–kaolin granules

Wheat-flour–kaolin granules were prepared using the methodology of Connick et al. (1991). Batches of 40 g of the formulation were prepared containing (a) microconidial solution (20 ml containing 1×10^7 CFUs ml^{-1} plus 3 ml sterile water), 32 g durum wheat-flour (DIVELLA, Italy), 6 g kaolin, and 2 g sucrose or (b) air-dried chlamydospore-rich biomass (2.1 g containing 2×10^8 CFUs g^{-1}, respectively) made up to 6 g with kaolin, 32 g durum wheat-flour, 2 g sucrose, and 23 ml sterile water.

All ingredients were kneaded with gloved hands until a cohesive dough formed which was subsequently passed through a small, hand-operated pasta maker. The sheets (1–1.5 mm thick) were air-dried on aluminium foil at 25 °C for two days. Dry sheets were ground and sieved to obtain the fraction that passed through a 2 mm mesh and collected on a 200 µm mesh. CFU g^{-1} were assayed in the formulated material after grinding by giving 0.1 g of the preparation with 10 ml sterile H_2O in a test tube. The sample was vortexed from time to time together with three glass beads (0.6 cm dia.) until disintegrated. 100-µl aliquots of appropriate dilutions were plated on half-strength PDA amended with 200 ppm chloramphenicol and 100 ppm streptomycin sulphate (three plates per each of three samples) and CFUs g^{-1} formulation were determined after incubation for three days at room temperature. The formulated material was stored in plastic bags at ambient laboratory conditions. The experiments were repeated with 23 ml microconidial solution containing 3.4×10^7 CFUs ml^{-1} or 1.7 g chlamydospore-rich biomass containing 2.5×10^8 CFUs g^{-1}, respectively.

CFUs were determined in two preparations containing chlamydospore-rich biomass (containing 1.2×10^7 and 2.2×10^7 initial CFUs g^{-1}, respectively) 2, 4, 6, 8, 10, and 12 months after formulation and in two microconidial preparations (containing 9×10^5 and 7×10^5 initial CFUs g^{-1}, respectively) 2, 4, 7, 8, 10, and 12 months after formulation.

Virulence of wheat-flour–kaolin granules in a pot experiment

A pot trial was set up in a greenhouse at a temperature regime of 25/15 °C (day/night) with supplemental light provided by HQLR-lamps (1000 W) for 13 h. Local field soil was mixed with sand (1 : 1, w/v) to provide a loamy sand which was steam-sterilised at 80 °C for 4 h to destroy alien weed seeds.

Plastic pots ($13 \times 13 \times 13\,cm^3$, with a capacity of 2 kg soil) were filled up to two-thirds of their height with soil. *O. cumana* seeds (50 mg, approximately 5000 seeds capable of germinating per kg of soil) were sprinkled onto the soil surface and mixed with the undercover soil-layer of 5 cm thickness with a spade. Wheat–kaolin granules containing microconidia (2.3×10^7 CFUs g^{-1}, hereinafter referred to as preparation P1) or chlamydospore-rich biomass (1.2×10^7 CFUs g^{-1}, hereinafter referred to as preparation P2) prepared as described above were incorporated along with the *Orobanche* seeds into the soil at the rates of 1 and 0.5 g per pot, respectively. Pots containing *Orobanche* seeds not inoculated with the fungus served as negative control (C−) and a fungus-free treatment without *Orobanche* seeds was set as a positive control (C+). The pots were entirely filled with soil and three sunflower seeds (cv. Iregi) were sown in each pot. The experiment was arranged in a completely randomised design with five replicates per treatment. Fourteen days after sowing, sunflower plants were thinned to one plant per pot and each pot was fertilised weekly with 20 ml of a 2% (v/v) Wuxal™ solution. When *Orobanche* shoots started to emerge, the number of emerged shoots and the proportion of shoots showing the typical disease symptoms (browning, bending over of shoots, and wilting) were determined weekly. At the end of the experiment (after 10 weeks) the above-ground sunflower dry matter was recorded after drying at 120 °C overnight. The experiment was repeated with *O. cumana* seeds originating from Turkey and two different batches of preparations P1 and P2 with 1.2×10^7 and 9.1×10^6 CFUs g^{-1}, respectively.

Statistical analysis

Statistical analysis was conducted using the combined data of replicated experiments when they had homogenous variances. Data analysis consisted of analysis of variance (ANOVA), the *t*-test for comparison of two means or Tukey's Studentised range test (HSD) for multiple comparisons. Percentage data were arcsine-transformed before analysis (Gomez and Gomez, 1984). All tests of significance were conducted at $P \leq 0.05$. When data were not normally distributed or showed heterogeneity of variances even after square-root or log-transformation, they were subjected to Kruskall–Wallis ANOVA on ranks. To determine significant differences of means, the Nemenyi test for multiple comparisons was used.

Results

Production of chlamydospores by F. oxysporum *f.sp.* orthoceras

Effect of different substrates on chlamydospore formation

All chlamydospores were of mycelial origin. The number of chlamydospores formed was lowest in sorghum stover alone and sorghum stover plus a mineral salt solution (Table 1). Adding yeast extract to the sorghum stover increased the number of chlamydospores, but only sorghum stover plus thick rye-based stillage enhanced the formation of chlamydospores significantly compared to sorghum stover alone. Adding 10% stillage increased the number of chlamydospores per ml approximately tenfold.

Effect of different straw and stillage types and their concentrations on conidiation and chlamydospore formation of F. oxysporum *f.sp.* orthoceras

Thirty percent (v/v) wheat-based stillage added to 1.5% (w/v) sorghum stover produced the greatest number of chlamydospores (Table 2). The use of maize straw and stillage was second best, although it did give inconsistent results. Of the stillage concentrations tested, the amendment of 30% (v/v) wheat-based stillage to the medium resulted in the highest chlamydospore counts

Table 1. Effect of different substrates on chlamydospore formation by *F. oxysporum* f.sp. *orthoceras*

Substrate	Number of chlamydospores ($\times 10^6$) per ml of medium
(a) Sorghum stover	0.8 (0.3) ab
(b) Sorghum stover + salt solution[1]	0.3 (0.1) a
(c) Sorghum stover + 2% celery extract[2]	1.7 (0.4) ab
(d) Sorghum stover + yeast extract[3]	3.5 (0.9) bc
(e) Sorghum stover + stillage (thin)[4]	1.5 (0.6) ab
(f) Sorghum stover + stillage (thick)[5]	8.5 (0.6) c

Values are the means of two experiments. Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Values in parentheses are SEs. All media contained 1.5% (w/v) very finely milled (<500 μm) sorghum stover and were made up to 100 ml with deionised H_2O.

[1] 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$ and 2 mg glucose per litre deionised H_2O; [2] prepared by autoclaving 20 g of celery in 100 ml deionised H_2O; [3] 1 g l^{-1} deionised H_2O; [4] 10 ml of rye-based stillage using only the supernatant; [5] 10 ml of rye-based stillage using the entire liquid.

Table 2. Effect of medium composition (stillage type, straw type, stillage and straw concentrations) on the formation of microconidia and chlamydospores by *F. oxysporum* f.sp. *orthoceras*

Treatment	Microconidia ($\times 10^8$)[1]	Chlamydospores ($\times 10^7$)[1]	
		Experiment I	Experiment II
(a) Stillage type (20%, v/v)			
Potato	n.d.[2]	0.6 (0.1) a	0.4 (0.1) a
Triticale	n.d.	0.6 (0.1) a	0.6 (0.1) a
Maize	n.d.	0.6 (0.2) a	1.7 (0.2) b
Wheat	n.d.	1.6 (0.2) b	1.9 (0.1) b
(b) Straw type (1.5% , w/v)			
Sorghum	1.8 (0.3) a	0.6 (0.1) a	1.5 (0.3) a
Maize	2.2 (0.3) a	0.6 (0.1) a	1.0 (0.2) ab
Wheat	1.8 (0.2) a	0.3 (0.03) a	0.5 (0.1) b
(c) Wheat-based stillage concentration, % (v/v)			
20	2.6 (0.2) a	1.1 (0.1) ab[3]	
30	2.2 (0.4) a	1.8 (0.2) b	
40	1.8 (0.3) a	1.4 (0.2) b	
50	2.8 (0.8) a	0.6 (0.3) a	
(d) Sorghum stover concentration, % (w/v)			
0	1.9 (0.1) a	0.7 (0.1) a[3]	
0.5	2.2 (0.1) a	0.8 (0.1) a	
1	2.1 (0.3) a	1.5 (0.2) b	
1.5	1.8 (0.4) a	2.3 (0.2) c	

Means followed by the same letter are not significantly different according to Tukey's HSD test or the Nemenyi test at the $\alpha = 0.05$ level of significance. Values in parentheses are SEs.
[1]Per ml of medium; [2]not determined; [3]values are the means of two experiments.

while less chlamydospores were formed as the stillage concentration increased. Since the highest sorghum stover concentration (1.5% (w/v)) yielded the highest chlamydospore counts, 2.5% as well as 3.5% (w/v) stover in the solution were also tested, but did not result in a significantly higher production of chlamydospores. No statistically significant effects of the tested media on microconidial production were recorded.

Effect of plant fibres on chlamydospore formation

The presence or absence of plant fibres represented by unfiltered and filtered medium, significantly affected chlamydospore formation. Filtered medium yielded only about 25% (1.5×10^6 chlamydospores ml^{-1}) of the chlamydospore counts that were recorded in the unfiltered medium (5.9×10^6 chlamydospores ml^{-1}).

Air-drying of biomass

Fresh fungal biomass contained 2.2×10^8 CFUs g^{-1}. This was corrected by the moisture content to 1.3×10^9 g^{-1}. Air-drying reduced the CFUs by 91% to

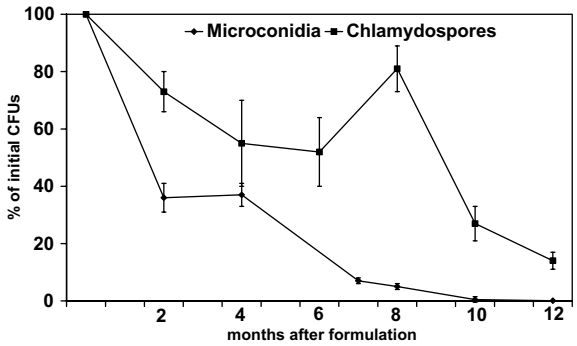

Figure 1. Effect of storage time on the CFUs in wheat-flour–kaolin granules containing different types of inocula (microconidia and chlamydospore-rich biomass). Values are the means of two experiments. Vertical lines indicate SEs.

1.2×10^8 g^{-1}. Air-dried material stored in plastic bags at room temperature maintained 53% of its initial CFUs after 10 months of storage.

Encapsulation of F. oxysporum *f.sp.* orthoceras *into wheat-flour–kaolin granules*

The wheat-flour–kaolin formulation procedure was suitable for *F. oxysporum* f.sp. *orthoceras*. The fungus was observed proliferating on the surface of the granules after 24–48 h exposure to favourable moisture conditions. Air-dried chlamydospore-rich biomass survived processing better than microconidia. In the final products, the microconidial preparations had lost about 80% of the initial CFUs and contained 1.1×10^6 or 3.2×10^6 CFUs g^{-1}, respectively, whereas 100% of the CFUs were still viable in the preparations containing chlamydospore-rich biomass which had a final content of 1.2×10^7 or 2.2×10^7 CFUs g^{-1}, respectively.

The stability of microconidial inoculum during storage at room temperature was inferior to that of the preparations containing chlamydospore-rich biomass (Figure 1). After two months of storage, the granules containing microconidia maintained only 36% of the initial CFUs. After seven months, the loss came up to already more than 90%. In the preparations containing chlamydospore-rich biomass, the CFUs steadily declined (with the exception of the sampling date eight months after formulation) to 14% of the initial CFUs after 12 months of storage.

Virulence of wheat-flour-kaolin granules in the greenhouse

Wheat-flour-kaolin granules controlled *O. cumana* in the greenhouse. Disease incidence as well as

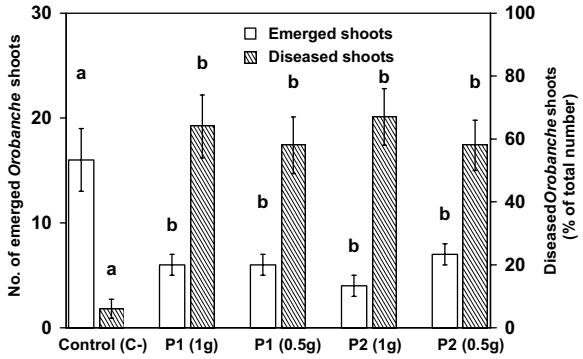

Figure 2. Effect of wheat-flour–kaolin granules containing different types of inocula and applied in different dosages (0.5 or 1 g per pot) on the total number and the proportion of diseased *O. cumana* shoots. Values are the means of two experiments. Multiple mean comparisons were performed among the numbers of emerged shoots using Tukey's HSD test and among the proportions of diseased shoots using the Nemenyi test at the $\alpha = 0.05$ level of significance. Bars with the same letter are not significantly different. Vertical lines indicate SEs. (C−) = sunflower plus *Orobanche*; P1 = granules containing microconidia; P2 = granules containing chlamydospore-rich biomass.

Orobanche emergence was significantly affected by both preparations compared to (C−) in both tested dosages (Figure 2). In all fungus-treated pots, 58–67% of the emerged *Orobanche* shoots showed disease symptoms compared to only 6% in the control treatment. No pronounced differences in efficacy were observed between the different types of inoculum. Sunflower biomass in the fungus-treated pots (data not shown) was not significantly increased compared to (C−).

Discussion

Plant extracts and residues affect chlamydospore formation in *Fusarium* spp. differently. Hildebrand and McCain (1978) observed that alfalfa straw, cotton seed meal, and soybean oil meal extracts increased chlamydospore formation. Huang et al. (1983) showed that a celery extract was the best medium for chlamydospore formation by *F. oxysporum* f.sp. *niveum*, while Hebbar et al. (1996) identified liquid media with amendments of soybean hull fibres or corn cobs as optimal for the chlamydospore formation by a mycoherbicidal *F. oxysporum* strain. Formation of chlamydospores by an isolate of *F. oxysporum* on sorghum stover was reported by Diarra et al. (1996) on solid medium and by Ciotola et al. (2000) in a two-stage fermentation process. In the present study, different additives to finely ground sorghum stover in a liquid suspension significantly influenced the numbers of chlamydospores formed. The stimulating effect of celery extract reported by Huang et al. (1983) was confirmed, but a sorghum stover/wheat-based stillage medium turned out to be the most suitable substrate: it was possible to produce at least 1×10^7 chlamydospores ml^{-1} in 1.5% sorghum stover and 30% wheat-based stillage within 16 days of fermentation. Hebbar et al. (1996) did not observe a reduced number of formed chlamydospores when their fibrous medium was filtered before fermentation. This is in contrast to the findings of this study, since the presence of plant fibres was important for the production of chlamydospores by *F. oxysporum* f.sp. *orthoceras*. Hebbar et al. (1996) reported reduced formation of mycelium and, consequently, of chlamydospores in media with a proportion of more than 1% solid amendment, which was not observed in this study either. The harvested biomass could be air-dried and stored at room temperature which makes the time of subsequent formulation less critical than in the case of wet inoculum which has to be processed immediately after harvesting.

Hildebrand and McCain (1978) claimed that a method with potential for large-scale production of inoculum should not require special equipment or handling and the inoculum produced should retain its potential for causing disease for long periods. The medium described here meets most of these criteria. However, the grinding of sorghum stover is time-consuming and labour-intensive. With regard to large-scale fermentation further investigations have to aim at replacing the straw with readily available fibrous material. Chlamydospore yield may be improved even after cultural conditions concerning light regime, aeration, pH, and temperature during the fermentation process, as well as the incubation period, have been optimised.

F. oxysporum f.sp. *orthoceras* can control *O. cumana* when propagules are encapsulated in wheat-flour–kaolin granules. The granules consist of biodegradable, inexpensive ingredients and can be produced on a commercial scale as was shown by Daigle et al. (1997) using twin-screw extrusion. The tested inocula reacted in a different way to the wheat-flour–kaolin formulation process. Microconidial inoculum lost approximately 80% of its initial CFUs while in the preparation containing air-dried chlamydospore-rich biomass (which had already lost the major part of its initial CFUs during

the first drying process), 100% of the initial CFUs were recovered. No significant difference in the efficacy of granules containing the different inocula was observed, however, the type of secondary propagules formed on the granules, which was not investigated, is probably more important for the infection process. Couteaudier and Alabouvette (1990) and de Cal et al. (1997) found a higher infectivity of chlamydospores than microconidia for different isolates of *F. oxysporum* on flax and tomato. In contrast, Bedi and Sauerborn (1998) showed that chlamydospores of *F. oxysporum* f.sp. *orthoceras* had a similar disease potential compared to microconidia when applied as freshly prepared inoculum. However, the authors introduced the inoculum directly into injured *Orobanche* shoots so that the advantages of chlamydospores that may lead to an increased infection potential, such as a higher ability of the germ tube to penetrate the plant (Silva-Muniz et al., 1991), were probably of lower importance for disease development.

Various factors such as water activity, storage temperature, or type and amount of the incorporated inoculum affect the viability of the living organisms in granular formulations during storage (Connick et al., 1997; 1998). In the study presented, the type of inoculum of *F. oxysporum* f.sp. *orthoceras* incorporated into wheat-flour–kaolin granules had a great influence on the stability of the formulated material at room temperature. The preparations containing air-dried chlamydospore-rich biomass maintained a higher proportion of their initial CFUs for a longer time than the microconidial formulations. The higher initial CFUs in the granules with chlamydospore-rich biomass than the microconidial preparations might be a reason for their better survival in storage. However, other experiments with wheat–kaolin granules containing microconidia (data not shown) showed that higher initial CFUs lead to a higher proportion of surviving propagules only during the first 4 months. In the experiments of Bedi and Sauerborn (1998) chlamydospores of *F. oxysporum* f.sp. *orthoceras* also retained their viability and virulence for a longer time during storage compared to conidia or mycelium. However, the better stability of formulations of chlamydospore-rich biomass is not necessarily due to the content of chlamydospores alone. Other reasons may be the different growth or nutritional stage of the fungal propagules or the additional content of mycelium. For example, Amsellem et al. (1999) observed a longer survival of formulated mycelium than conidia of a mycoherbicidal strain of *F. oxysporum*. The ingredients of the broth that was formulated together with the liquid inoculum or the substrate particles in chlamydospore-rich biomass could have influenced the survival of the propagules as well.

The smallest dosage of wheat-flour–kaolin granules used in the conducted pot experiments was 0.5 g per pot which can be extrapolated to about 300 kg granules/ha. This is an improvement compared to the 800 kg inoculum/ha used by Bedi (1994) in a field experiment achieving approximately the same efficacy of control. However, for practical relevance, the applied amount will have to be reduced. In-furrow application might be an option to decrease the inoculum amount.

Future investigations should try to improve the wheat-flour–kaolin formulation, e.g. by adding different amendments, and compare it to that of other formulations of the fungus.

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