The Use of Various Substrates for Large-Scale Production of Fusarium oxysporum f. sp. cannabis Inoculum

D. C. Hildebrand and A. H. McCain

Department of Plant Pathology, University of California, Berkeley, CA 94720.

This research was supported in part by U.S. Department of Agriculture, Agricultural Research Service Grant 12-14-100-9974 (34).

Accepted for publication 26 January 1978.

ABSTRACT

HILDEBRAND, D. C., and A. H. MCCAIN. 1978. The use of various substrates for large-scale production of Fusarium oxysporum f. sp. cannabis inoculum. Phytopathology 68:1099-1101.

Chlamydospore formation was enhanced in *Fusarium oxysporum* f. sp. *cannabis* in an aqueous solution individually amended with beef extract, soytone, proteose peptone #3, glycine, β-alanine, succinate, lactate, or $NaNO_3$. Chlamydospore formation was less with glucose, $(NH_4)_2SO_4$, tyrosine, and tryptone. The ideal defined medium for chlamydospore formation was a solution of glycine-succinate-$NaNO_3$. Diffusates from alfalfa straw, cottonseed meal, and soybean oil meal induced chlamydospore formation, but few spores were formed with diffusates from sugar beet pulp, barley straw, or safflower meal. Large-scale inoculum production by the fungus was achieved by culturing the fungus on a mixture of barley straw plus either the glycine-succinate-$NaNO_3$ solution, alfalfa straw, cottonseed meal, or soybean meal. Chlamydospores produced on the glycine-succinate-$NaNO_3$-barley straw substrate retained their disease potential over a 6-mo period at room temperature.

Additional key words: Cannabis sativa, biological control.

The biological control of undesirable plants by using plant pathogens is just beginning to be explored. Our investigations into the use of fungi to control hemp *(Cannabis sativa)* suggested that *Fusarium oxysporum* f. sp. *cannabis* would be an ideal pathogen to employ because it is very host specific and will persist for long periods in soil. In order to use this fungus for biological control, it was necessary to develop methods for the production of large amounts of *F. oxysporum* inoculum on an economical basis. The ideal inoculum of this fungus probably should be chlamydospores because those are its natural survival structure. This paper reports on the effect of various nutritional substrates on the formation of chlamydospores by *F. oxysporum* f. sp. *cannabis* and how certain substrates can be applied to the large-scale production of inoculum.

MATERIALS AND METHODS

Two clones of *F. oxysporum* f. sp. *cannabis* isolated from hemp growing in the vicinity of Caserta, Italy, were used. Stock cultures of the fungus were maintained on potato-dextrose agar slants and periodically checked for virulence.

Chlamydospore induction.—Chlamydospore formation was tested in 250-ml Erlenmeyer flasks containing 50 ml of various test solutions. Each flask was seeded with 0.1 ml of an aqueous conidia + mycelium suspension of the fungus taken from 3- to 4-wk-old cultures, and maintained at 28 C in the dark.

The number of chlamydospores produced per milliliter of test solution after 4 days of incubation was determined by direct microscopic counts of acid fuchsin-stained spores retained on Nuclepore membrane filters. Longer incubation periods did not appreciably change the number of chlamydospores observed except when natural substrates such as soybean meal, cottonseed meal, or alfalfa straw were used.

Propagule abundance in soil.—The number of propagules per gram of soil was determined with the method of Nash and Snyder (5).

Inoculum effectiveness measurement.—The ability of the inoculum to cause disease was measured by dipping the roots of 10- to 12-day-old *C. sativa* seedlings into a water suspension of a succinate-glycine-$NaNO_3$-barley straw inoculum mixture (see Inoculum Production section for composition), planting the inoculated seedlings in a sand-peat potting mixture (2), and maintaining them in a growth chamber with an average temperature of 24 C. The average number of days for a seedling to die was considered to be a measure of the effectiveness of the inoculum. Twelve seedlings were inoculated in each trial.

RESULTS

Chlamydospore formation on artificial substrates.—Preliminary observations were made on the effects of diverse substrates, individually or combined, in stimulating chlamydospore formation. These substrates included L-lysine, L-glycine, L-proline, L-asparagine, β-alanine, L-tyrosine, thiamine, nicotinic acid, biotin, choline, ascorbic acid, glucose, succinate, D-tartaric acid, L-lactic acid, gluconic acid, m-inositol, mannitol, $(NH_4)_2SO_4$, $NaNO_3$, beef extract, proteose peptone #3, soytone, and lecithin. Of these, glycine, β-alanine, succinate, lactate, $NaNO_3$, beef extract, proteose peptone

00032-949X/78/000193$03.00/0

TABLE 1. Influence of various carbon and nitrogen sources upon chlamydospore formation by *Fusarium oxysporum* f. sp. *cannabis*

	Glucose		Succinate		Lactate	H_2O
	0.01%	0.1%	0.01%	0.1%	0.1%	
$NaNO_3$ (0.1%)	3.2×10^4[a]	1.1×10^4	NT	4.1×10^4	4.1×10^4	0.0
Glycine (0.1%)	0.0	0.0	18.2	24.1	NT	0.0
Glycine (0.1%) + $NaNO_3$ (0.1%)	28.6	2.6	19.8	50.0	42.1	16.0
Glycine (0.1%) + $(NH_4)_2SO_4$ (0.1%)	NT	0.8	NT	15.3	NT	0.0
H_2O	0.0	0.0	0.0	0.0	0.0	0.0

[a]Chlamydospores formed per flask after 4 days of incubation in the dark at 28 C. Average of two experiments with two replications per experiment. NT = not tested.

#3, and soytone appeared to enhance chlamydospore formation whereas others such as glucose, $(NH_4)_2SO_4$, tyrosine, and tryptone were unable to support spore formation. Consequently, the effects of glucose, succinate, lactate, glycine, and $NaNO_3$, and various combinations thereof on chlamydospore formation were examined in more detail.

The most efficient substrate for induction of chlamydospore formation was a mixture of succinate-glycine-$NaNO_3$ (Table 1), followed by the lactate-glycine-$NaNO_3$ mixture. Apparent glucose repression of chlamydospore formation was observed in the glycine-$NaNO_3$-glucose mixture by noting that it was less at the higher level of glucose than at the lower level, or the complete absence of glucose. The chlamydospores were formed from hyphae from germinated macroconidia after apparent secession of mycelial growth.

Chamydospore formation induction on natural substrates.—The wide variations in numbers of spores formed with the various peptones suggested that wide variations also might occur with natural substrates. Consequently, diffusates from various plant products were tested for ability to induce chlamydospore formation. The diffusates were prepared by autoclaving 75 mg of the plant product in 50 ml of distilled water at 121 C for 15 min and decanting the supernatant liquid into 250-ml Erlenmeyer flasks. Flasks were inoculated with 0.1-ml suspensions of the fungus as previously described and incubated for 2 wk.

As with the peptones, the various plant products differed considerably in their induction of chlamydospore formation (Table 2). Diffusates from alfalfa straw was the best for chlamydospore formation followed by those from cottonseed meal and soybean meal. Few chlamydospores were formed in the milo, oat, or almond-hull diffusates with only slightly more being formed in the presence of dried sugar beet pulp, barley straw, or safflower meal diffusates.

Inoculum production.—Ease of handling inoculum as well as effectiveness were factors considered while developing the inoculum production methods. Barley straw was selected as a relatively inert diluent of the substrates used to induce chlamydospore formation; few chlamydospores form when barley straw alone is used to grow the fungus. The final mixtures tested consisted of 800 g of barley straw and 2 liters of distilled H_2O plus 160 g of soybean meal, alfalfa straw, or cottonseed meal. Another mixture that was tested consisted of 160 ml of a 0.1% Na succinate-0.1% glycine-0.1% $NaNO_3$ solution plus 40 g of barley straw. All mixtures were autoclaved twice at 121 C for 15 min before being inoculated with the fungus. Incubation was for 3 wk in large (2- to 3-liter) glass flasks at room temperature with the inoculum then being air-dried at room temperature for 14 days upon removal from the flasks. Survival of the fungus was measured by adding the inoculum to soil at a rate of 10 g/1,000 g of soil and determining the number of propagules in soil after 5 wk.

Results of this test indicated that all straw-substrate mixtures tested were suitable for the production of inoculum. The cottonseed oil meal mixture was the best with 3.50×10^4 propagules/g soil detected, whereas 2.67×10^4 propagules/g soil were detected for the alfalfa mixture, 2.55×10^4 propagules/g soil for the succinate-glycine-$NaNO_3$ mixture, and 1.36×10^4 propagules/g soil for the soybean mixture.

Inoculum effectiveness stability.—Air-dry inoculum stored in plastic bags at room temperature remained fully effective for 6 mo. The number of days for seedlings to die under conditions of the test ranged 18 to 20 days. At 9 mo some loss in disease potential was noted and after 1 yr considerable loss was noted, the average number of days for seedling death was 24 and 30, respectively.

TABLE 2. Number of chlamydospores of *Fusarium oxysporum* f. sp. *cannabis* formed in diffusates prepared from various plant sources

Substrate	Chlamydospores formed[a] after:	
	1 wk ($\times 10^4$)	2 wk ($\times 10^4$)
Safflower meal	0.0[a]	8.5×10^4
Beet pulp	0.1	4.3
Cottonseed meal	10.4	90.0
Almond hulls	0.6	0.1
Barley straw	0.6	6.7
Soybean meal	2.2	24.0
Milo (rolled)	0.4	0.4
Oat straw	0.2	1.2
Alfalfa straw	11.9	191.7
H_2O	0.0	0.0

[a]Chlamydospores formed per flask after incubation in an unlighted incubator at 28 C. Average of two experiments, two replications per experiment.

DISCUSSION

The formation of chlamydospores, the natural survival unit of *F. oxysporum,* is affected by a number of factors (1, 3, 4, 6, 7). Our findings confirm that different nutritional substrates markedly affect chlamydospore formation. Specific amino acids are important as indicated by the differences in chlamydospore formation occurring with the various peptones as well as the amino acids tested. These nutritional effects probably also explain why the various natural plant products affected chlamydospore formation differently as the plant products would all have a different nutritional composition.

The capacity of certain nutrients to induce chlamydospore formation in *F. oxysporum* was used as the basis for the development of a method with the potential for large-scale production of the fungal inoculum. Ideally, such a method should be relatively simple, require no special equipment or handling, and the inoculum produced should retain its potential for causing disease for long periods without special handling. Our inoculum production methods using both natural plant products and a specific nutritional combination meet these criteria. These methods have been used to produce over 20 kilograms of inoculum used successfully in field trials.

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