Effects of Isolation Techniques and Media on the Differential Isolation of *Fusarium* species

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ABSTRACT

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Three isolation techniques, plating sieved debris, plating root pieces, and soil plating were tested in conjunction with three isolation media (Martin's rose bengal, Komada, and Nash-Snyder) for recovery of *Fusarium* spp. from soil. Comparisons among isolation techniques and media were based on recovery of 2,057 isolates representing 17 species plus two varieties of *Fusarium*. Recovery of individual species of *Fusarium* was dependent on the type of isolation technique used, but generally was not dependent on the medium used. There were significant interactions between isolation techniques and media. Diversity indices of the *Fusarium* spp. recovered were highest with the debris technique and with Martin's rose bengal medium. Determination of the presence and abundance of a *Fusarium* spp. in soil is dependent on appropriate isolation procedures.

Additional key words: grassland soil, inoculum potential, soil survey.

Fusarium spp. persist in soil as conidia, chlamydospores, or mycelium. Certain isolation methods may influence which particular propagative unit, and thus which species, will grow (27). Caution has been urged in comparing surveys of soilborne fusaria because the isolation methods used may impose a bias on the kinds of species found and their frequency of occurrence. However, most surveys of soilborne fusaria have been based on a single isolation technique, the dilution plate technique (3,7,8,12,13,16,17,34), or Warcup's (29) soil plate technique (19). Several studies have involved the use of two isolation techniques (10,11,25,33).

Estimates of populations of individual formae speciales within a species may also be affected by the isolation technique used. Smith and Snyder (25) found that *F. oxysporum* f. sp. *vasinfectum* (Atk.) Snyd. and Hans. persisted in barley fields rotated with cotton. This pathogen accounted for 28.9% of the Fusarium isolates detected by using the soil dilution plate technique and 92% of the Fusarium isolates from soilborne plant fragments of barley and weed species (25). Thus, the dilution plate technique indicated a much lower inoculum potential of *F. oxysporum* f. sp. *vasinfectum* in the soil (3,130 propagules per gram of soil) than in plant fragments (85,000 propagules per gram of fragments).

Fungus species have various nutritional requirements (18,20,28,30,32); therefore, the medium used for isolation may determine which species are isolated. Selective media are available for isolation of one or more *Fusarium* spp. (9,16,18,28). However, comparative studies of the effect of media on the recovery of a broad spectrum of *Fusarium* spp. from soil have not been reported. Three media were tested for recovery of fusaria from corn stalks, but no differences were reported (5). Kreutzer (11) reported no suppression of growth of nine species of *Fusarium* on Nash-Snyder medium.

The objectives of this study were to determine whether there are differences in the recovery and diversity of *Fusarium* spp. isolated from soil by testing all combinations of three isolation techniques and three isolation media. A preliminary report has appeared (15).

MATERIALS AND METHODS

Sites and sampling. Soil samples were collected from four North Dakota grassland sites. The description of these sampling sites and corresponding soil analysis information are given in Table 1. Sites 1 and 2 were sampled twice during the growing season in 1979 and three times each during 1980 and 1981. Sites 3 and 4 were sampled once, at midseason, in 1980 and 1981.

For each site and sampling date, five to eight soil cores were collected along a line transect (at 1-m intervals and to a depth of 12 cm) and bulked. These composite samples were placed in sterile plastic bags, transported in an ice chest, and stored at 3 C until processed within 3 wk. Prior to bioassay, a sample was taken from each composite soil for soil nutrient, moisture, and pH analysis by the North Dakota State University Soil Testing Laboratory (Table 1).

Isolation techniques. For each composite sample, three isolation techniques were tested for recovery of *Fusarium* spp.: plating of sieved debris, plating of root pieces, and soil plating. Debris was sieved following the procedure described by Tio et al (27), except that a 0.295-mm-mesh sieve was substituted for their 0.05-mm-mesh sieve. One hundred twenty pieces of debris ranging in size from 0.3 mm to 1 cm in length and 0.3–5 mm in diameter were cultured per composite sample. Debris was identified as nonliving plant tissue, but no attempt was made to identify individual fragments.

Root pieces approximately 50 mm × 0.5–1.0 mm were cut from intact root systems found in the soil samples, washed in sterile distilled water for 5 min, dipped in 1% NaOCl for 5 sec, rinsed in sterile distilled water, damp dried on paper toweling, and plated four pieces per plate, 120 pieces per composite sample. No attempt was made to define the specific plant origin of the roots.

Soil was bioassayed by Warcup's soil plate method (29) in 1979. In 1980 and 1981 this method was modified so that 5 mg of soil was distributed evenly over the surface of the solidified media, rather than incorporating 10 mg of soil into the molten agar. This was done by sprinkling the soil over the medium from a sieve made by drilling 14 evenly distributed 1.7-mm-diameter holes in the bottom of a plastic petri dish. This modification facilitated separation and recovery of colonies. Thirty soil plates were prepared per composite sample.

Our preliminary studies (15) included use of the standard dilution plate technique, but results indicated that the same kinds and numbers of *Fusarium* spp. were isolated in soil plates as in dilution plates. Because dilution plates required much more time, labor, and materials than soil plates, use of the former was discontinued.

Isolation media. For each composite sample, the three isolation

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techniques were tested on three media: Martin's rose bengal agar (14), a general purpose medium for cultivation of soil fungi; Nash-Snyder medium (16), modified by the addition of 120 ppm neomycin; and Komada medium (9). The last two are selective for *Fusarium* spp. Preliminary tests (15) included potato-dextrose agar for an all purpose medium. Use of this medium was discontinued because contamination was too great to allow recovery of all colonies of Fusarium.

Identifications. All plates were incubated at 20–24 C for 7–10 days at 0.4–0.5 m below a combination of warm white (F40WW-T12, Norelco Corp., Hightstown, NJ 08520) and black light (F40-BLB, Sylvania Corp., Elk Grove, IL 60007) fluorescent illumination with a 12-hr photoperiod. These temperatures were selective for the mesophilic component of the population. All Fusarium colonies and fungal colonies of uncertain affinity were then transferred to potato-dextrose agar slants. Identifications were based on morphology of conidia developed on carnation leaf agar (4) and morphology of conidia and conidiophores produced on slide mounts (23). Isolates were identified according to Gordon (6–8). His taxonomic system was based on the study of Fusarium isolates from cereal seeds and soils of cereal plots and grasslands of temperate regions. In addition, we recognized *Fusarium sulphureum* Schlect. sensu Booth (1) (= *F. sambucinum* Fuckel f-6 Wollenw.).

Data analysis. To measure the likelihood of recovery of a species by an isolation technique or medium, frequency values were determined. Frequency of a species was defined as the number of plates containing that species per isolation technique or medium. A single colony or many colonies of the same species in the same plate scored a frequency of one. Thus, frequency values describe whether or not a technique or medium will result in recovery of a species, and these values are independent of the magnitude of that recovery.

To measure the magnitude of recovery of a species by isolation technique or medium, relative densities were determined. Relative density or percent occurrence of a species was defined as the number of isolates of a species divided by the total number of isolates of *Fusarium*.

The independence of recovery of species by techniques or media used was tested using chi-square contingency table analyses (26) of frequencies. The possibility of technique by media interactions was also tested in the same manner.

To measure the diversity of species recovered by each isolation technique and medium Simpson's Index (24) of diversity was used. Diversity was calculated as follows:

$$\text{Diversity} = 1 - \text{Simpson's Index}$$
$$\text{Simpson's Index} = \sum_{i=1}^{s} p_i^2$$

p_i = relative density of each species

s = total number of species recovered.

This diversity index was used because it includes two components: species richness (number of species) and species equitability (relative abundance of a species). Simpson's Index values range from $1/s$ to 1. If every isolate was a different species, the index would equal $1/s$ and be very small, indicating maximum diversity; if all the isolates were the same species the index would equal 1, indicating minimum diversity.

RESULTS

***Fusarium* species isolated.** For all years, sites, techniques, and media, 17 species plus two varieties of *Fusarium* were isolated. Of 2,057 isolates of *Fusarium* the relative density of each species was as follows: *F. acuminatum* Ell. & Ev. (12.0%), *F. avenaceum* (Fr.) Sacc. (8.1%), *F. concolor* Rg. (0.2%), *F. culmorum* (W. G. Smith) Sacc. (0.2%), *F. equiseti* (Cda.) Sacc. (14.0%), *F. graminearum*

TABLE 1. Sampling site descriptions and their soil analyses

Characteristics[a]	Site 1	Site 2	Site 3	Site 4
Plant community Dominant plants	Sandhills tall-grass prairie *Andropogon hallii*[b] Hack.	Grazed mixed-grass prairie *Bouteloua gracilis*[b] Lag. ex Steua.	Ungrazed mixed-grass prairie *Bouteloua gracilis*[b] and *Stipa* L. spp.	Cultivated field: Fallow, 1979 Wheat, 1980 Flax, 1981
Soil type	Mixed, frigid Udipsamment	Sandy, mixed Udorthentic Haploboroll	Fine-loamy, mixed Typic Haploboroll	Fine-loamy, mixed Typic Haploboroll
Soil moisture (%)	3.4	3.8	20.0	20.4
Soil pH	7.4	7.2	6.8	6.5
Nitrate-nitrogen (ppm)	1.8	1.4	2.4	29.4
Phosphorous (ppm)	7.0	4.0	2.0	6.0
Potassium (ppm)	71.0	83.0	305.0	312.0

[a] All numerical characteristics are 3-yr averages and were determined from the soil samples collected for isolation.
[b] *Andropogon hallii* = sand bluestem, *Bouteloua gracilis* = blue grama grass, *Stipa* spp. = needle grasses.

TABLE 2. Interactions of *Fusarium* spp. by isolation techniques, species by media, and techniques by media, in relation to years and sites of tests

		Degrees of freedom and probability values of chi-square tests for interaction[a]					
		Species × techniques[b]		Species × media[c]		Techniques × media	
Year	Sites	df	P	df	P	df	P
1979	1,2	12	0.0001	12	NS[d]	4	0.0111
1980	1,2,3,4	16	0.0001	16	0.0295	4	NS
1981	1,2,3,4	12	0.0002	12	NS	4	0.0123
1979–1981	1	16	0.0001	16	NS	4	0.0072
1979–1981	2	16	0.0002	16	NS	4	0.0875
1980–1981	3	14	0.0001	14	NS	4	0.0582
1980–1981	4	12	0.0092	12	NS	4	NS
All years and sites		20	0.0001	20	NS	4	0.0029

[a] Tests were made with species having frequency values >5.
[b] Isolations were made from debris (2,400 pieces total), roots (2,400 pieces total), and soil (600 plates).
[c] Media used were Komada, Martin's rose bengal, and Nash-Snyder (600 plates per medium).
[d] NS = probability level >0.06.

TABLE 3. Contingency table for species by isolation technique interactions using the combined 3-yr data, over all sites

Fusarium species[b]	Observed and expected frequency values for each technique and the totals[a]						
	Debris[c]		Root[c]		Soil plate[c]		
	Observed	Expected	Observed	Expected	Observed	Expected	Totals
acuminatum	*109*[d]	77	30	42	*59*	80	198
avenaceum	59	53	*15*	30	63	55	137
equiseti	64	68	36	37	77	71	177
graminearum	10	9	3	5	11	10	24
moniliforme	10	15	11	8	18	16	39
oxysporum	*205*	245	*161*	134	268	255	634
oxysporum var. *redolens*	*7*	17	*20*	9	17	18	44
poae	8	7	3	4	8	7	19
reticulatum	19	14	5	7	11	14	35
sambucinum	*25*	14	*0*	8	12	15	37
solani	37	33	18	18	31	35	86
Totals	553		302		575		1,430

[a] Chi-square = 87.97**; 20 df.
[b] Species with total frequency values ≤ 5 were not included in the chi-square analysis.
[c] A total of 1,800 plates were examined, 600 per technique.
[d] Values in italics indicate sources of significant contribution to the chi-square value.

TABLE 4. Diversity indices of isolation techniques and isolation media used in recovery of *Fusarium* spp. from soil

Year[b]	Diversity indices[a]					
	Isolation techniques			Isolation media		
	Debris	Root	Soil plate	Martin's	Komada	Nash-Snyder
1979	0.82	0.57	0.68	0.79	0.68	0.70
1980	0.72	0.69	0.67	0.70	0.68	0.71
1981	0.76	0.62	0.68	0.75	0.73	0.68
Total[c]	0.77	0.66	0.68	0.75	0.68	0.69

[a] Diversity index = 1 − (Simpson's Index) in which Simpson's Index = $\Sigma_{i=1}^{s} p_i^s$ (p_i = relative density of each species. s = total number of species recovered).
[b] Each year's value is based on data over all sites sampled that year.
[c] Totals are calculated from 3 years of data.

Schwabe (1.2%), *F. lateritium* Nees em. Snyder & Hansen (0.2%), *F. merismoides* Corda (0.2%), *F. moniliforme* Sheld. (2.6%), *F. moniliforme* Sheld. var. *subglutinans* Wr. & Rg. (0.6%), *F. oxysporum* Schlect. em. Snyder & Hansen (48.8%), *F. oxysporum* Schlect. var. *redolens* (Wr.) Gordon (2.3%), *F. poae* (Pk.) Wr. (1.0%), *F. reticulatum* Mont. (2.0%), *F. sambucinum* Fuckel (2.0%), *F. semitectum* Berk. & Rav. (0.1%), *F. solani* (Mart.) App. & Wr. em. Snyder & Hansen (4.3%), *F. sporotrichioides* Sherb. (0.2%), and *F. sulphureum* Schlect. (0.1%). Of 1,800 plates prepared, 1,470 contained one or more *Fusarium* spp.

Isolation techniques. Of the 2,057 Fusarium isolates, 33.3% were isolated from debris, 18.6% from roots, and 48.1% from soil plates. *Fusarium* spp. were not recovered independently of the isolation plating technique used. Interactions between species and techniques occurred regardless of year or site analyzed (Table 2). For the debris technique recovery of *F. acuminatum* and *F. sambucinum* was greater than expected while recovery of *F. oxysporum* and *F. oxysporum* var. *redolens* was less than expected (Table 3). A greater than expected recovery of *F. oxysporum* and *F. oxysporum* var. *redolens* and a less than expected recovery of *F. avenaceum* and *F. sambucinum* occurred with isolation from root pieces. For the soil plate technique recovery of *F. acuminatum* was less than expected. These significant interactions were determined through chi-square contingency table analysis of frequencies, using the combined 3-yr data (Table 3). The 11 species or varieties having a total frequency greater than five were analyzed. The other species were too infrequent for inclusion in a valid chi-square test. A similar analysis of the density values, which measured magnitude of recovery, showed a highly significant chi-square value and the same contributions to significance as seen in Table 3.

Diversity indices (Table 4) for each year and for the combined 3-yr data show that isolating from debris consistently gave the highest diversity index and that isolations from root pieces averaged the lowest. Figure 1A–C shows the relative frequency of the major *Fusarium* species recovered by each isolation technique for the combined 3-yr data. *F. oxysporum* was the most abundant species isolated by all three techniques. However, for *F. oxysporum*, the share of the total was smallest with the debris technique and greatest with the root piece technique.

Isolation media. The percent recovery of species by each medium was 47.8% for Komada, 9.0% for Martin's, and 43.3% for Nash-Snyder. Generally, no medium tested enhanced or inhibited the likelihood of isolating any one species. Seven of eight chi-square analyses of frequency values for media by species interactions were nonsignificant (Table 2). However, similar analyses of density values gave significant chi-square values for the individual years and the combined 3-yr data. Thus, the choice of medium affected magnitude of recovery of a species more so than likelihood of recovery. In these instances there was primarily a greater than expected recovery of *F. acuminatum* with Martin's rose bengal medium.

Martin's rose bengal medium gave the highest diversity indices (Table 4). Figure 1D–F shows the relative frequency of recovery of the major *Fusarium* spp. by each medium tested. The most frequent species recovered by any medium was *F. oxysporum*. However, for *F. oxysporum*, the share of the total was the smallest on Martin's rose bengal medium.

Isolation techniques × media interactions. Chi-square contingency table tests for interaction between isolation techniques and media were significant in five of eight analyses (Table 2). The source of significance in these five analyses was primarily due to greater than expected recovery from root isolations when the medium was Martin's rose bengal agar.

DISCUSSION

The recovery of certain species of *Fusarium* was significantly and consistently influenced by the isolation technique and not by the variable site factors (Table 1) or sampling year (Table 2). Thus, use of only one isolation technique may fail to provide the optimum conditions for recovery from soil of a maximum number of *Fusarium* spp. or of an individual species. Also, population levels of some species may be inadequately estimated if only one isolation technique is used. These data confirm the opinion that isolation techniques do bias Fusarium soil survey results (2,27).

Parkinson et al (21) suggested that it is impossible to devise a

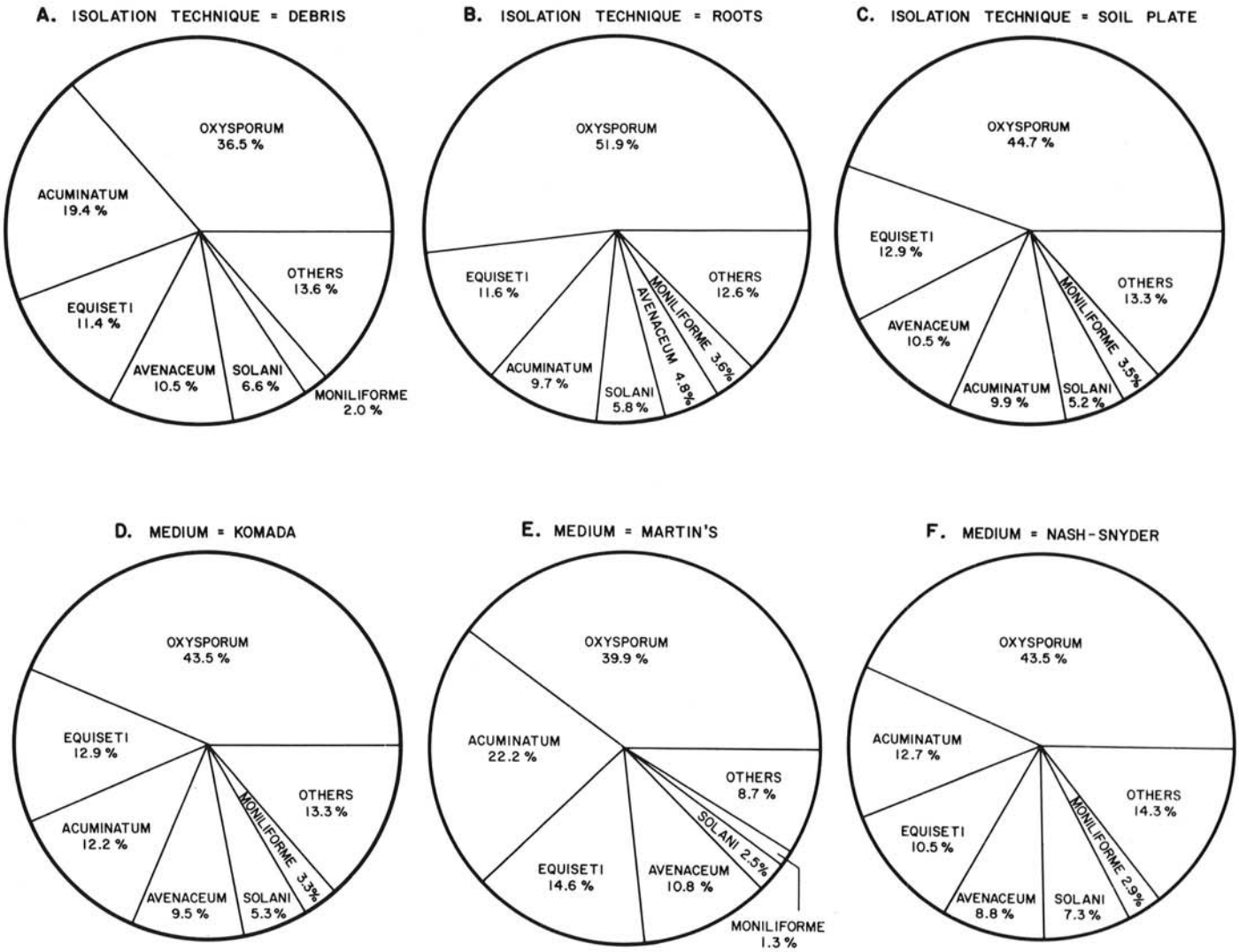

Fig. 1. The frequency of recovery of individual *Fusarium* spp. among isolation techniques including **A,** debris, **B,** roots, and **C,** soil, and among media including **D,** Komada, **E,** Martin's rose bengal, and **F,** Nash-Snyder. The portion of the pie charts designated as "others" represents the 11 species and two varieties isolated infrequently.

single isolation technique by which all fungal forms present in the soil can be isolated, since any isolation technique is somewhat selective. Because different *Fusarium* spp. occupy different habitats in the soil, the isolation techniques used here were aimed at recovering species within these various habitats. The debris plating technique was used to recover those species associated with washed organic matter. Parkinson and Kendrick (22) recovered a greater frequency of *Fusarium* spp. from washed organic matter (50%) than washed mineral soil (30%) or unwashed soil (4%). Use of root pieces was aimed at recovering *Fusarium* spp. associated with intact root systems of plants from the sites. The soil plate technique was used to isolate the fast growing fungal component from soil, existing primarily as spores (20,29,31), and because it allows for rapid screening of a large number of soil samples (20). Warcup (29,31) showed this technique to isolate more species per plate and per soil sample than the standard dilution plate method.

It is difficult to assess whether the recovery of species with very low frequencies is favored or inhibited by any one technique or medium. Thus, in any study involving the isolation of *Fusarium* spp., more than one technique and medium should be used.

Fungi such as *Trichoderma*, *Penicillium*, *Rhizopus*, and *Aspergillus* were prevalent on Martin's rose bengal medium and their presence inhibited recovery of colonies of *Fusarium*, particularly with the soil isolation technique. The significant interaction between the root isolation technique and Martin's rose bengal medium suggests that this medium would enhance recovery of *Fusarium* spp. from roots. The Martin's rose bengal medium may have been less inhibitory than Komada or Nash-Snyder media to the fusaria growing slowly from the inner tissues of the surface-disinfected root pieces. Diversity indices indicated that more species are more frequently isolated by use of the debris technique and Martin's rose bengal medium, and this accounts for the corresponding lower frequencies of *F. oxysporum* (Fig. 1A and E).

These results document that use of a single isolation technique or medium may insufficiently represent the distribution and abundance of *Fusarium* spp. in soil. Improper conclusions concerning the presence and abundance of a *Fusarium* spp. may be avoided by use of a combination of isolation techniques and media that increase frequency, density, and diversity of species recovered. The use of such a combined approach by researchers working with *Fusarium* would help to establish inoculum potential of a species or forma specialis as well as facilitate comparison of survey results.

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