

An In Vitro Assay to Evaluate Sources of Resistance in *Asparagus* spp. to Fusarium Crown and Root Rot

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ABSTRACT

Stephens, C. T., and Elmer, W. H. 1988. An in vitro assay to evaluate sources of resistance in *Asparagus* spp. to Fusarium crown and root rot. *Plant Disease* 72:334-337.

Asparagus spp. grown aseptically in test tubes containing Hoagland's solution and agar were inoculated with spore suspensions of pathogenic isolates of *Fusarium oxysporum* f. sp. *asparagi* or *F. moniliforme*. Disease severity was assessed after 3 wk, using a scale based on percentage of root tissue infected as evidenced by lesions. Species of *Asparagus* susceptible to *F. o. f. sp. asparagi* were used to determine the optimal spore concentration for disease development and to evaluate the virulence of several Michigan isolates of *F. o. f. sp. asparagi* and *F. moniliforme*. Inoculations with 1 ml of a conidial suspension (10^6 conidia per milliliter) of *F. o. f. sp. asparagi* or *F. moniliforme* produced consistently high disease ratings on susceptible seedlings. Disease reactions of resistant and susceptible species of *Asparagus* in the in vitro assay were compared with those obtained from transplants in greenhouse evaluations. Seedlings of *A. sprengeri* and *A. myersii* were rated as resistant in the in vitro assay, whereas *A. plumosus* and *A. setaceus* 'pyramidalis,' *A. palestinus*, *A. stipularis*, and all cultivars of *A. officinalis* were susceptible. This method represents a rapid, relatively inexpensive technique for evaluating *Asparagus* spp. germ plasm for resistance to Fusarium crown and root rot.

Asparagus (*Asparagus officinalis* L.) fields in Michigan and other areas are being removed from production prematurely because of asparagus decline syndrome (1,2,9-12). The disease syndrome is characterized by reduced production from declining plant densities and reduced size of spears. Fusarium root and crown rot is incited by *Fusarium oxysporum* (Schlecht.) emend. Snyder & Hans. f. sp. *asparagi* Cohen & Heald and *F. moniliforme* (Sheld.) emend. Snyder & Hans., the major causes of asparagus decline (2,9,10,12). Both pathogens were consistently isolated from all commercial asparagus plantings surveyed in Michigan (11; Stephens, unpublished).

Strategies for controlling Fusarium crown and root rot of asparagus are limited by the perennial nature of asparagus plantings. Soil fumigation is ineffective after a few years (14,16) because of a rapid reintroduction of the pathogen from contaminated seeds (4), crowns (2), and wind-dispersed soils from neighboring fields, which can harbor both pathogens as natural residents (Stephens, unpublished). In addition, it is difficult to alter soil pH and

crown depth over time in a perennial crop. Fusarium crown and root rot of asparagus is more severe in fields with acid soils and when crowns are planted shallowly (10-15 cm deep) (11). Likewise, injuries resulting from cultivation practices further predispose crowns to Fusarium infection (18).

Development of resistant cultivars appears to be the most viable long-term strategy for control, but no highly resistant cultivars have been developed (7,15). In fact, most asparagus cultivars currently available are selections made from the old varieties derived from asparagus seeds originally introduced in the United States in the early 1900s. Recently released cultivars such as Jersey Centennial (Rutgers-Mich Hy 202) (8) possess increased vigor and presumably greater tolerance to Fusarium crown and root rot, but all cultivars have been found to be susceptible (Stephens, unpublished). The California asparagus hybrid U.C. 157 used in this study was derived from heterozygous diecious parents selected from open-pollinated plants. Therefore, these hybrids are not homogeneous and thus have substantial genetic variation. No true F_1 asparagus hybrids are currently available (7,21).

A program was initiated at Michigan State University to develop methods to evaluate resistance to Fusarium crown and root rot in a global collection of *Asparagus* spp. germ plasm. Field and greenhouse screening techniques were unacceptable because of large space requirements, labor costs, and inconsistent results resulting from cross-contamination (19). Therefore, we adopted and modified

an in vitro assay developed by Davis (6) and later used by Damicone and Manning (5) for assaying pathogenicity of *Fusarium* spp. isolates on asparagus seedlings.

The objectives of this research were to test the usefulness of in vitro assay for screening *Asparagus* spp. using different isolates of *F. o. f. sp. asparagi* and *F. moniliforme* and to compare the results from the in vitro assays with those of greenhouse trials.

MATERIALS AND METHODS

Preparation of seedlings. Seeds of U.C. 157 were supplied by the University of California Seed Foundation. Asparagus cultivar Mary Washington, *A. sprengeri* Regel., *A. plumosus* Bak., *A. myersii* Hort., and *A. setaceus* 'pyramidalis' (Kunth) Jessop were supplied by Ball Seed Co. (West Chicago, IL). Seeds of *A. stipularis* Forsk and *A. palestinus* Bak. were collected from mature wild plants in Israel. Taiwanese selection No. 2 was provided by C. C. Tu (21). Previous work in our laboratory demonstrated that U.C. 157 is susceptible to *F. o. f. sp. asparagi* and *A. sprengeri* is resistant (19).

Seeds of *Asparagus* spp. were surface-disinfested in 1% sodium hypochlorite (20% household bleach) for 30 min, rinsed with sterile distilled water, placed in beakers containing 100 ml of acetone plus 2.5 g of benomyl (5 g of Benlate 50WP), covered, and stirred with a magnetic stirrer bar for 18-20 hr. The benomyl-acetone treatments were effective in eradicating the *Fusarium* spore contaminants that persist deep within seed crevices (4). Seeds were washed twice with 100 ml of acetone and twice with sterile distilled water, then air-dried for 5-10 min under sterile conditions. Seeds were germinated on water agar (0.6%, w/v). Five to 7 days later, single germinated seeds with the radicle just emerging were aseptically placed into 15 × 2.5 cm diameter test tubes containing 20 ml of solidified sterile Hoagland's solution No. 2 plus 0.6% agar (pH 6.8) (3), and the tubes were sealed with plastic caps. Any tubes that became contaminated were discarded. Clean tubes with seedlings were incubated at 25 C 10-15 cm under cool-white fluorescent tubes for 16-hr photoperiods.

Preparation of inoculum and inoculation procedure. Three isolates each of *F. o. f. sp. asparagi* (F-10, FOA4, FOA5)

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Journal article 12318 of the Michigan State Agricultural Experiment Station.

Accepted for publication 4 November 1987 (submitted for electronic processing).

and *F. moniliforme* (F-12, FM49, FM57) were obtained from diseased asparagus crowns and stems from different areas of Michigan. Pieces of diseased tissue (5 × 5 mm) were cut from the plant, surface-disinfested in 0.5% sodium hypochlorite (10% household bleach) for 1 min, rinsed in sterile distilled water, and plated on Komada's selective medium (KM) (13). Emerging colonies that resembled *F. oxysporum* or *F. moniliforme* were singled-spored onto potato-carrot agar (22) and identified by spore morphology (17). Isolates were tested for pathogenicity on highly susceptible U.C. 157 in the in vitro assay described below. An isolate of *F. o. f. sp. apii* race 2 (FA-3) pathogenic to celery, but not to asparagus, and a nonpathogenic isolate of *F. oxysporum* (FO70) that had been recovered from a peach orchard soil were included as nonpathogenic controls in different experiments. Isolates were maintained in soil storage (17).

Inocula were increased on potato-carrot agar (22) and incubated at 21–23 C for 10 days. Conidia were washed with sterile distilled water from the surface of the plates through four layers of sterile cheesecloth and centrifuged at 3,000 g for 5 min. The resuspended pellet consisted of macroconidia and microconidia at a ratio of 1:25–1:50, depending on the isolate. Serial dilutions of conidia were adjusted to yield 10⁴, 10⁵, 10⁶, or 2 × 10⁶ microconidia and macroconidia per milliliter.

When asparagus seedlings had begun to develop secondary roots (10–14 days), 1 ml of the spore suspension (10⁶ conidia per milliliter unless otherwise noted) of the test isolate was placed on top of the agar into each of 10 replicate tubes and incubated as described above for 3 wk. Our procedure differed from that of Davis (6) in that established healthy seedlings were inoculated with a conidial suspension instead of germinated seeds being placed on an agar medium previously colonized by the pathogen. Seedlings were rated for disease development by visual examination of the root system and assignment of a disease rating on a 1–5 scale modified from that of Damicone and Manning (5), where 1 = no disease, 2 = lesions present on 0–25% of the root system, 3 = lesions on 25–50% of the root system, 4 = lesions on 50–75% of the root system, and 5 = lesions on 75–100% of the root system. All experiments were repeated three times with similar results.

Greenhouse trials. Seeds of U.C. 157 (susceptible) and *A. sprengeri* (resistant) were disinfested in 1% sodium hypochlorite (20% household bleach) for 30 min and germinated on sterile moistened filter paper in glass petri plates at 22–24 C. Vigorous seedlings were selected 7–10 days later and transplanted into 72-cell (2.5 × 2.5 cm) plastic flats containing a commercial potting soil (1:1:1, v/v,

vermiculite:peat:perlite, pH 5.2). Each plant received approximately 20 ml of Peter's soluble fertilizer (20-20-20, 15 g/L, W. R. Grace Co.) per week for 2 mo. Healthy plants were transplanted into 10-cm-diameter clay pots containing the potting mix described above and grown for another month. Each plant received 100 ml of the same fertilizer every 2 wk.

At 13–14 wk of age, crowns of U.C. 157 and *A. sprengeri* were transplanted into 12.5-cm-diameter clay pots containing approximately 1.1 kg of steamed soil mix (2:1, v/v, sand:greenhouse soil) supplemented with 8 g of air-dried millet seed inoculum. This inoculum was prepared by autoclaving the millet seed with water (2:1, v/v, millet seed:water) twice for 1 hr on consecutive days and seeding with agar plugs colonized by *F. o. f. sp. asparagi* and *F. moniliforme* and incubated for 3 wk. There were 10 replicate pots containing one plant each, with an equal number of plants growing in soil with uncolonized sterile millet seed serving as a controls. Plants were rated 8 wk later on a disease scale of 1–5, where 1 = no root lesions or vascular discoloration in roots, crown, or stems; 2 = fewer than five root lesions but no vascular discoloration in roots, crown, or stems; 3 = five to 10 root lesions with slight vascular discoloration and fewer shoots and roots than the control; 4 = more than 10 root lesions with vascular discoloration in the roots, crown, and stems and sparse shoots and feeder roots; and 5 = heavily rotted crown or death of the plant. This disease rating scale described more accurately the symptoms commonly seen with older infected greenhouse transplants than the scale used for infected seedlings. Therefore, disease ratings could not be compared directly.

Statistical procedures. Treatments in the in vitro assays and in the greenhouse consisted of 10 replicates. Data were subjected to an analysis of variance (ANOVA), and treatment means were compared by Duncan's multiple range test at *P* = 0.05. Although the differences in rating scales in the in vitro assay and

the greenhouse trials hindered a valid statistical comparison, the scales do parallel one other in describing the disease expression of the laboratory seedlings and greenhouse plants. Therefore, an *F* test of significance was computed (*P* = 0.01) between the two methods as general test of agreement between the data.

RESULTS

Virulence assays in vitro. All isolates of *F. o. f. sp. asparagi* caused root lesions on U.C. 157 (Table 1). Isolate FOA5 incited significantly more root lesions than isolate F-10, with isolate FOA4 ranked between them. All isolates of *F. moniliforme* caused disease, but isolates FM49 and FM57 incited significantly more lesions than isolate F-12. Reddish brown elliptical lesions became evident on primary and secondary roots of U.C. 157 10 days after inoculation in test tubes, but ratings were made after 3 wk to allow sufficient time for disease to develop in all treatments. When disease reactions were severe, large necrotic lesions developed on the primary root but not on secondary roots. This condition usually resulted in a water-soaked, flaccid primary root (Fig. 1). Chlorosis and tip necrosis of the young fern tissue frequently, but not always, preceded death of the seedling, but fern tissue of less-diseased seedlings was still green after the incubation period. *A. sprengeri* had little or no disease when inoculated with *F. o. f. sp. asparagi* or *F. moniliforme*. *F. o. f. sp. apii* race 2 (FA-3) caused no lesions on either asparagus species. All succeeding experiments were conducted with *F. o. f. sp. asparagi* isolate FOA5 and *F. moniliforme* isolate FM57.

Effect of inoculum concentration of pathogenic and nonpathogenic isolates of *F. oxysporum* on asparagus seedlings in vitro. Mean disease ratings of U.C. 157 seedlings inoculated with isolate FOA5 were 1.0, 2.0, 3.6, 4.4, and 4.3, respectively, with inoculum concentrations of 0, 10⁴, 10⁵, 10⁶, and 2 × 10⁶

Table 1. Disease ratings of *Asparagus officinalis* 'U.C. 157' and *A. sprengeri* seedlings inoculated with isolates of *Fusarium oxysporum* f. sp. *asparagi*, *F. o. f. sp. apii* race 2, and *F. moniliforme* in agar supplemented with Hoagland's salt solution

<i>Fusarium</i> spp.	Isolate	Disease ratings ¹	
		U.C. 157	<i>A. sprengeri</i>
Noninoculated	...	1.0 a ²	1.0 a
<i>F. o. f. sp. apii</i> race 2	...	1.0 a	1.0 a
<i>F. o. f. sp. asparagi</i>	F-10	1.5 a	1.0 a
	FOA4	2.8 b	1.0 a
	FOA5	3.7 bcd	1.1 a
<i>F. moniliforme</i>	F-12	3.0 bc	1.2 a
	FM49	4.0 d	1.2 a
	FM57	4.2 d	1.2 a

¹Disease rating scale: 1 = no disease, 2 = lesions present on 0–25% of the root system, 3 = lesions on 25–50% of the root system, 4 = lesions on 50–75% of the root system, and 5 = lesions on 75–100% of the root system.

²Values based on the mean of 10 seedlings: values followed by different letters are significantly different according to Duncan's multiple range test at *P* = 0.05.

Table 2. Reactions of *Asparagus* spp. to inoculation with *Fusarium oxysporum* f. sp. *asparagi* and *F. moniliforme* in the in vitro assay

<i>Asparagus</i> spp.	Disease rating ¹		
	Noninoculated	<i>F. o. f. sp. asparagi</i>	<i>F. moniliforme</i>
<i>A. officinalis</i>			
U.C. 157	1.0 a ²	3.7 cd	3.9 cd
Mary Washington	1.0 a	3.0 bc	3.0 bc
Taiwanese selection No. 2	1.1 a	2.0 ab	2.4 b
<i>A. plumosus</i>	1.0 a	2.4 b	2.2 ab
<i>A. sprengeri</i>	1.1 a	1.1 a	1.5 ab
<i>A. setaceus</i> 'pyramidalis'	1.0 a	3.0 bc	3.0 bc
<i>A. myersii</i>	1.0 a	1.2 a	1.8 abc
<i>A. palestinus</i>	1.0 a	3.3 bcd	3.1 bc
<i>A. stipularis</i>	1.0 a	4.5 d	4.6 d

¹ Disease rating scale: 1 = no disease, 2 = lesions present on 0–25% of the root system, 3 = lesions on 25–50% of the root system, 4 = lesions on 50–75% of the root system, and 5 = lesions on 75–100% of the root system.

² Values based on the mean of 10 seedlings; values followed by different letters are significantly different according to Duncan's multiple range test at $P = 0.05$.

Table 3. Comparison of disease ratings of *Asparagus officinalis* 'U.C. 157' (susceptible) and *A. sprengeri* (resistant) using the in vitro assay and greenhouse screening tests for resistance to *Fusarium* crown and root rot

<i>Fusarium</i> spp.	Disease rating			
	In vitro ¹		Greenhouse ²	
	<i>A. officinalis</i>	<i>A. sprengeri</i>	<i>A. officinalis</i>	<i>A. sprengeri</i>
Noninoculated	1.1 a ³	1.0 a	1.2 a	1.7 a
<i>F. o. f. sp. asparagi</i>	3.7 b	1.1 a	2.8 b	1.2 a
<i>F. moniliforme</i>	4.2 b	1.3 a	5.0 c	1.8 a

¹ Disease rating scale: 1 = no disease, 2 = lesions present on 0–25% of the root system, 3 = lesions on 25–50% of the root system, 4 = lesions on 50–75% of the root system, 5 = lesions on 75–100% of the root system.

² Disease rating scale: 1 = no root lesions or vascular discoloration in roots, crown, or stems; 2 = fewer than five root lesions but no vascular discoloration in roots, crown, or stems; 3 = five to 10 root lesions with slight vascular discoloration and fewer shoots and roots than the control; 4 = more than 10 root lesions with vascular discoloration in the roots, crown, and stems and sparse shoots and feeder roots; 5 = heavily rotted crown or death of the plant.

³ Values followed by different letters are significantly different according to Duncan's multiple range test at $P = 0.05$. In vitro assay (MSE = 0.35) vs. greenhouse trials (MSE = 0.23) not significant by F test at $P = 0.01$.

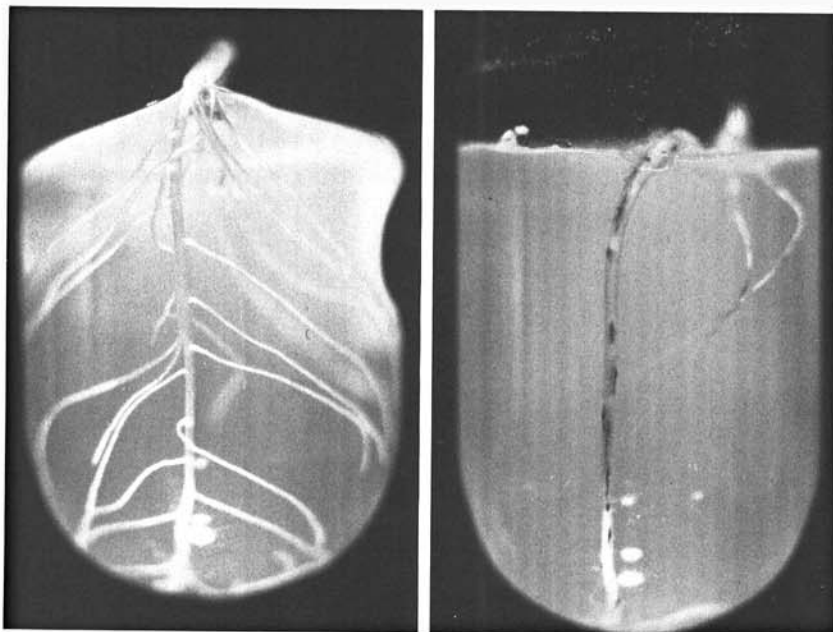


Fig. 1. (Left) Roots of healthy asparagus seedling (U.C. 157) growing in Hoagland's agar. (Right) Seedling (U.C. 157) inoculated with *Fusarium oxysporum* f. sp. *asparagi* showing reddish brown lesions on a water-soaked, flaccid primary root and severely restricted secondary root development.

conidia per milliliter. Root lesions were detected in only a few seedlings inoculated with 10^4 conidia per milliliter. No disease escapes occurred at 10^5 conidia per milliliter. The numbers of lesions and extent of their development were higher at 10^6 but were not significantly different from those at 2×10^6 conidia per milliliter. No root lesions or phytotoxic effects were detected on asparagus seedlings treated with the nonpathogenic *F. oxysporum* isolate FO70.

Disease evaluations of *Asparagus* spp. in the in vitro assay. The highly susceptible asparagus cultivars U.C. 157 and Mary Washington and *A. setaceus* 'pyramidalis,' *A. palestinus*, and *A. stipularis* received high disease ratings (3.0–4.6) when inoculated with either pathogen (Table 2). Taiwanese selection No. 2 of *A. officinalis* and *A. plumosus* were less severely diseased, but most lesions were found only on certain individual seedlings of both species. Seedlings of *A. sprengeri* and *A. myersii* had the lowest disease ratings, except that *F. moniliforme* inoculations on *A. sprengeri* and *A. myersii* produced disease ratings not significantly different from those on Taiwanese selection No. 2 and *A. plumosus*.

Comparison of disease evaluations from in vitro and greenhouse inoculations. No significant difference existed between the in vitro assay and the greenhouse method when both data sets were compared by an F test at $P = 0.01$, but this comparison must be viewed with caution, as different ratings scales were used (Table 3). Severe disease developed in the highly susceptible U.C. 157 in the in vitro test and in greenhouse inoculations. *F. moniliforme* caused significantly more disease on U.C. 157 in greenhouse tests (mean disease rating = 5.0) than did *F. o. f. sp. asparagi* (mean disease rating = 2.8), but these differences were not observed in the in vitro assay. Less disease was observed on *A. sprengeri* in the in vitro assay (mean disease rating = 1.1) and in the greenhouse tests (mean disease rating = 1.2) after inoculation with *F. o. f. sp. asparagi*. A few root lesions were evident on *A. sprengeri* when inoculated with *F. moniliforme*. Repetitions of these inoculations in the in vitro assay indicated that *F. moniliforme* was more virulent than *F. o. f. sp. asparagi* on *A. sprengeri*. Root infections were also detected on noninoculated *A. sprengeri* plants grown in the greenhouse. *F. moniliforme* was isolated from root pieces plated on KM. This inoculum was thought to have been introduced by splashing contaminated soil.

DISCUSSION

Davis (6) developed and utilized the in vitro assay to study the selective pathogenicity of different formae speciales of *F. oxysporum* isolates on

eight different hosts (asparagus not included). In his procedure, seedlings were grown in agar previously colonized by different formae speciales of *F. oxysporum*. In our studies, 2- or 3-wk-old seedlings were established in agar before inoculation so that comparisons between treatments represented differences in the disease resistance levels of established seedlings with secondary roots. Our method also alleviated other possible interferences such as phytotoxic metabolites that could affect the growth of the germinated seed in the colonized agar medium.

Our in vitro technique detected differences in the disease reactions of various *Asparagus* spp. to *F. o. f. sp. asparagi* and *F. moniliforme* that were similar to reactions of older plants in the greenhouse. The lack of a significant difference between the disease evaluation data from the in vitro assay and the data derived from the greenhouse trials indicates that the in vitro assay can be used to evaluate resistance in *Asparagus* spp. to *Fusarium* crown and root rot. However, the use of different disease ratings in the two methods may have affected the test statistic.

The more resistant Taiwanese selection No. 2 was distinguished from the highly susceptible cultivar U.C. 157 in the in vitro assay. The few diseased individuals of Taiwanese selection No. 2 (21) and *A. plumosus* may indicate that these species were segregating for resistance to *Fusarium* crown and root rot, but no discernible pattern of segregation was detected in our trials. *A. sprengeri*, *A. myersii*, and *A. plumosus* are produced as open-pollinated ornamental crops in which no known cultivars exist; these data, therefore, represent evaluations on heterogeneous individuals. Since *A. sprengeri* and *A. myersii* are morphologically very different from *A. officinalis* and the other *Asparagus* spp. examined, it is not presently known whether hybridization of *A. sprengeri* or *A. myersii* with *A. officinalis* is possible.

There were significant statistical differences between the relative virulence of the isolates of *F. o. f. sp. asparagi* and

F. moniliforme. The virulence of isolate FOA5 was considerably less to greenhouse-grown asparagus crowns than to seedlings in the in vitro assay. This may have resulted from the use of two different disease rating scales, but younger plants have been reported to be more susceptible than older crowns to *F. o. f. sp. asparagi* (10).

Many pathogenic formae speciales of *F. oxysporum* cause disease on only one host genotype (20). Our data suggest that the host specificity is much wider in *F. o. f. sp. asparagi* than in most other formae speciales. Five of seven *Asparagus* spp. gave susceptible reactions to Michigan isolates of *F. o. f. sp. asparagi* and *F. moniliforme*, indicating that susceptibility to *Fusarium* crown and root rot must be common in *Asparagus* spp.

The in vitro assay has been used in our laboratory to evaluate large numbers of plants with greater control of environmental parameters (Stephens, unpublished). Evaluations can be taken without disturbing root systems, which can result in poor recovery of badly infected roots. In addition, the frequent contamination from splashing soil and airborne conidia of *F. moniliforme* seen in greenhouse trials was eliminated. Use of the in vitro system has provided a rapid screening technique for assaying asparagus germ plasm with minimal time, space, and effort.

ACKNOWLEDGMENTS

We thank Rene De Vries and Sally Garrod for their technical assistance.

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