In Vitro Assay of Excised Cotyledons of Alfalfa (*Medicago sativa*) to Screen for Resistance to *Colletotrichum trifolii*

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ABSTRACT

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An in vitro assay using excised cotyledons of alfalfa has been developed that accurately detects resistance to *Colletotrichum trifolii*. The adaxial surfaces of excised cotyledons are placed on sterile moist filter paper in petri plates. The cotyledons are sprayed with a spore suspension, and resistant and susceptible reactions can be determined after incubation for 14 days at 24 C with a 16-hr light period. Susceptibility is based on the presence of sporulating acervuli. Five isolates of *C. trifolii* reacted similarly in the assay, and the range of effective inoculum was 2,500–10,000 spores per spray. Excised cotyledons and the seedlings from which the cotyledons were obtained were simultaneously tested in vitro and in a greenhouse. Saranac AR and Saranac had 86.5 and 96.2% agreement of observations, respectively, between the two screening techniques. A blind test of six cultivars was done, and the percent resistance obtained with the in vitro screen agreed with published values. Results indicate the usefulness of this technique as an alternative to greenhouse screening methods.

Anthracnose, caused by *Colletotrichum trifolii* Bain & Essary, has caused significant disease losses in alfalfa (*Medicago sativa* L.) (3,5). Several workers (2,4,9) have shown that selection for resistance is successful, and a number of cultivars with high levels of resistance are now available.

Several methods have been developed to screen and evaluate seedlings for resistance. The most commonly used method consists of spray-inoculating flats of seedlings with conidia and incubating them, first in a moist chamber and then in a greenhouse or laboratory growth chamber (9). Morrison (7) modified this method by using seedling boxes, which provided reactions similar to those obtained with inoculation chamber methods. Graham et al (6) developed a laboratory technique for cocultivating seedlings and fungus on cornmeal agar and transferring the survivors to a greenhouse. All of the techniques are destructive in that the susceptible seedlings do not survive selection. More recently, Ostazeski and Elgin (11) described a nondestructive method for inoculating stems to evaluate their resistance to *C. trifolii*.

This project was designed to rapidly evaluate seedling resistance in a nondestructive manner that neither introduced *C. trifolii* into the greenhouse nor contaminated a clean laboratory with greenhouse soil. We found that excised cotyledons of seedlings, inoculated in vitro, would express resistance to *C. trifolii*. Various factors were tested to determine their influence on resistance expression, and results of in vitro responses were related to a greenhouse screen.

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MATERIALS AND METHODS

Plant material. In all experiments, the cultivars Saranac and Saranac AR were used as susceptible and resistant controls, respectively. Plants were grown from seed either in the laboratory or in the greenhouse. For laboratory-grown plants, seeds were sown in a plastic sterilizing pan (46 × 38 cm) (Nalge Co., Sybron Corp., Rochester, NY) with 2.5–5.0 cm of sand covering the bottom and watered as needed with one-half-strength Hoagland's solution. The trays were enclosed in a clear autoclave bag and sealed with tape. A 0.2-μm Acrodisc filter (Gelman Scientific, Inc., Ann Arbor, MI) was connected at the opposite end and attached to an Optima model aquarium pump (Hazen Corp., Mansfield, MA) for continuous airflow. Plants were grown at room temperature under lights (about 1,000 ft-c) for 10 days.

Greenhouse-grown plants were planted in plastic trays (45 × 45 cm) in Jiffy Plus Mix. Ten rows of seeds were planted per tray with either 35 or 50 seeds per row. When only the two standard cultivars were used, seeds were sown in alternating rows. Plants were grown under ambient greenhouse conditions for 10 days.

Fungal isolates. Five isolates of *C. trifolii* were used in this project. Isolate F54A from California and isolate F56A from Colorado were obtained by direct isolation in our laboratory. Isolates F55A and F59A were provided by W. Thyr, USDA, University of Nevada, Reno, and isolate F60A (ATCC 32358) was obtained from the American Type Culture Collection, Rockville, MD. Cultures of all isolates were derived from single spores at the inception of this project and were stored at 4 C on potato-dextrose agar (PDA) slants (12).

Inoculum was produced by immersing a 1-cm^2 plug of a sporulating culture in 1–2 ml of sterile water and shaking gently. Two-tenths of a milliliter of the spore suspension was transferred aseptically to PDA plates and spread evenly over the surface. Plates were incubated for 6 days at 24 C with a 16-hr light period (about 1,000 ft-c). The spore suspension for inoculation was made by serially flooding several culture plates with 15 ml of sterile water and shaking gently. Spore suspensions were counted and adjusted to the desired concentration with a hemacytometer.

Inoculations. A sterile filter paper (Whatman No. 1) was placed in either glass or plastic petri plates (100 × 15 mm), and 1.5 ml of sterile distilled water was added to each plate. Cotyledons from 10-day-old seedlings were excised as close to the stem as possible, and the adaxial surface was placed on the prepared plates. Inoculation was performed by spraying an equal volume of liquid onto the surface with either an airbrush (Type

H, Paasche Airbrush Co., Chicago, IL) or a chromist spray unit (Gelman Scientific, Inc.). Plates were held at a 45-degree angle 5 cm from the tip of the sprayer. Each spray was timed and a volume of 60 μl containing 5,000 spores was delivered. A cotyledon was centered in the spray pattern each time, and the surface of the cotyledon received a uniform pattern of spore deposition. After inoculation, plates were sealed with Parafilm and incubated under the same conditions as described for inoculum. The viability of the spore suspension was checked for each experiment by spray-inoculating PDA plates and counting the percent germination after 18–24 hr.

Scoring and evaluating resistance. Cotyledons were observed with a dissecting scope (×15) 4, 7, 9, 11, and 14 days after inoculation. Most other screening methods commonly score whole-plant symptoms on a numerical severity scale of 1–5 (2,7–9). The evaluation we used for cotyledons is a more stringent modification of those schemes. A susceptible rating was given to any cotyledon showing sporulation on the surface. A cotyledon was scored resistant if it remained green and appeared healthy throughout the 2-wk period. Percent resistance was used as the final criterion for evaluating resistance (1,4,7).

Several experiments were done to determine which factors influenced the in vitro assay. In all of the experiments, each treatment consisted of six to 15 plates with 20 cotyledons per plate. Controls (usually two plates) were made for each treatment by inoculating cotyledons with sterile water. For statistical analysis, each plate was considered a replicate.

Experiment 1: inoculum density. Six spore densities (500, 2,500, 5,000, 7,500, 10,000, and 50,000 spores per 60 μl) were compared. As described before, these values refer to the number of spores released in each spray.

Experiment 2: isolates. The five isolates of *C. trifolii* described earlier were tested individually and as a combination of all five isolates.

Experiment 3: predisposition of the host by growth conditions. Preliminary experiments were done with laboratory-grown plants. Eventual comparison with a greenhouse screen required a comparison of laboratory- and greenhouse-grown plants. Cotyledons were collected from plants grown under both conditions and compared directly.

Experiment 4: correlation of in vitro and greenhouse screens. Correlation of the two screening techniques was made by simultaneously using the same plant in both screens. Cotyledons were excised from greenhouse-grown seedlings and placed in a specific pattern in plates and inoculated as described before. The remaining seedling was labeled so that the resistance reaction of the cotyledon and seedling could be compared on an individual basis. The greenhouse screen was done as described previously (8,9). The experiment was conducted in a randomized complete block design and repeated twice.

Experiment 5: blind cultivar trial. To test the usefulness of this technique for cultivars with unknown levels of resistance, a "blind" cultivar test was done. Six cultivars with known and different levels of resistance were obtained as coded samples. The cultivars were Arc, Saranac AR, Vancor, Raidor, WL 314, and Saranac. The experimental percent resistance values were obtained and compared with the known levels of resistance. Resistance categories and ranges of percent resistance established by the National Certified Alfalfa Variety Review Board (from publications of the Association of the Official Seed Certifying Agencies during 1973–1981) were used for comparisons (Table 1). This experiment was conducted twice.

RESULTS

The differential responses of the five isolates on certain cultivars indicated that all of the isolates were race 1 of *C. trifolii* (1,4,10). The mean percent spore germination for all experiments was 85.1 ± 7.4% with a range of 72.5–93.7%.

Rating a cotyledon susceptible on the basis of the presence of sporulation proved to be a useful criterion for evaluation. Susceptible cotyledons showed a range of responses by the end of the incubation period. The responses ranged from small areas of sporulation (0.5 cm^2) to complete degradation of the cotyledon with profuse sporulation (Fig. 1).

The resistant reaction criterion (green and healthy-appearing cotyledons) required slight modification. Most of the cotyledons remained green, but about 30% had various degrees of necrotic spotting (Fig. 1). Some cotyledons with more extensive necrosis also showed limited sporulation. Previously published work using a severity scale of 1–5 would rate this sort of reaction as a 2 and consider it resistant (8,9). In this study, these cotyledons were rated susceptible on the basis of the presence of sporulation. Another consideration in evaluation was the cut end of the cotyledon. Occasionally, there was a minute amount of sporulation at the cut surface although the rest of the cotyledon was healthy. If there was collapse of the tissue more than 5 mm from the cut surface, the cotyledon was rated susceptible. These two events led to stricter requirements for a resistant rating. In some cases, the known resistant controls showed lower final percent resistance with this evaluation scheme.

The general trend in a response of a cultivar was to decline rapidly during the first week of incubation and then level off within 10–14 days to a final percent resistance that was characteristic for the cultivar (Figs. 2 and 3). The susceptible and resistant control cultivars (Saranac and Saranac AR) responded as expected in five experiments (an average of 1.33 ± 1.19 and 61.3 ± 8.5% resistance, respectively).

Experiment 1: inoculum density. The susceptible control, Saranac, showed virtually no resistance to *C. trifolii* at inoculum densities greater than 500 spores per spray (Table 2). Similarly, Saranac AR showed considerable resistance at all concentrations except the highest one tested (50,000 spores per spray). This supports Ostazeski and Elgin's (11) findings of a wide response to inoculum for these cultivars. All subsequent experiments were done at 5,000 spores per spray.

Experiment 2: isolate comparison. All five isolates, as well as a combination of these isolates, reacted similarly in the in vitro assay (Fig. 2). An analysis of variance indicated that there was no

Table 1. Mean percent resistance of six alfalfa cultivars to *Colletotrichum trifolii* in two experiments using the in vitro cotyledon assay

Cultivar	NCAVRB expected values[v]	Percent resistance[w] Exp. 1	Percent resistance[w] Exp. 2	Determined resistance level[x]
Arc	50% (HR)	71.3 a	65.0 a	HR
Saranac AR	50% (HR)	60.5 b	46.3 b	HR
Vancor	30–50% (R)	44.4 c	43.6 b	R
Raidor	15–30% (MR)	46.2 c	20.6 c	R
WL 314	5–15% (LR)	10.6 d	4.3 d	LR
Saranac-1[y]	5% (S)	51.9 c	53.9 b	HR
Saranac-2	5% (S)	NI[z]	0.0 d	S
Saranac-3	5% (S)	NI	0.0 d	S

[v] NCAVRB = National Certified Alfalfa Variety Review Board expected percent resistance ranges and subsequent resistance classification for each cultivar: HR = high resistance, R = resistance, MR = moderate resistance, LR = low resistance, and S = susceptible.

[w] Each value is the mean of seven to nine replicates in each experiment. A replicate is a petri plate with 20 cotyledons per plate. Numbers followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

[x] Designated resistance category based on the average of the two means from both experiments.

[y] This cultivar was mislabeled as Saranac.

[z] NI = not included.

difference between isolates ($P = 0.05$). Isolate F56A was used in all further experiments because of its abundant sporulation.

Experiment 3: predisposition of host by growth conditions. The two environments for growth of the host before inoculation did not significantly alter the resistance response in the in vitro assay ($P = 0.05$) (Fig. 3). This validated earlier results obtained with laboratory-grown plants.

Experiment 4: correlation of in vitro and greenhouse screens. Data from this experiment were analyzed in two ways. First, the cotyledon and the paired seedling were compared on an individual basis and a percent agreement (no. of similar observations/total no. of observations × 100) between the two screens was determined. Second, an analysis of variance was done on two factors: cultivar and screening method. In the first experiment, percent agreement of observations for the cultivars Saranac and Saranac AR was 95.2 and 84.5%, respectively. In the second experiment, 97.2 and 88.4% of the observations agreed for Saranac and Saranac AR, respectively. When combined, 91.3% of the observations ($n = 759$) were in agreement. The analysis of variance of the mean percent resistance (Table 3) also indicated that there is no significant difference between the techniques ($P = 0.05$).

Experiment 5: blind cultivar trials. In the first run of this experiment, Saranac-1 gave an unexpectedly high percent resistance value (51.9%) (Table 1). When the experiment was repeated, two separate sources (Saranac-2 and Saranac-3) of Saranac seed were tested. In the second experiment, the separate sources (Saranac-2 and Saranac-3) of Saranac seed were both scored susceptible. This indicates that Saranac-1 was actually another cultivar that was mislabeled. The determined percent resistance was within the expected ranges for each cultivar, with the exception of

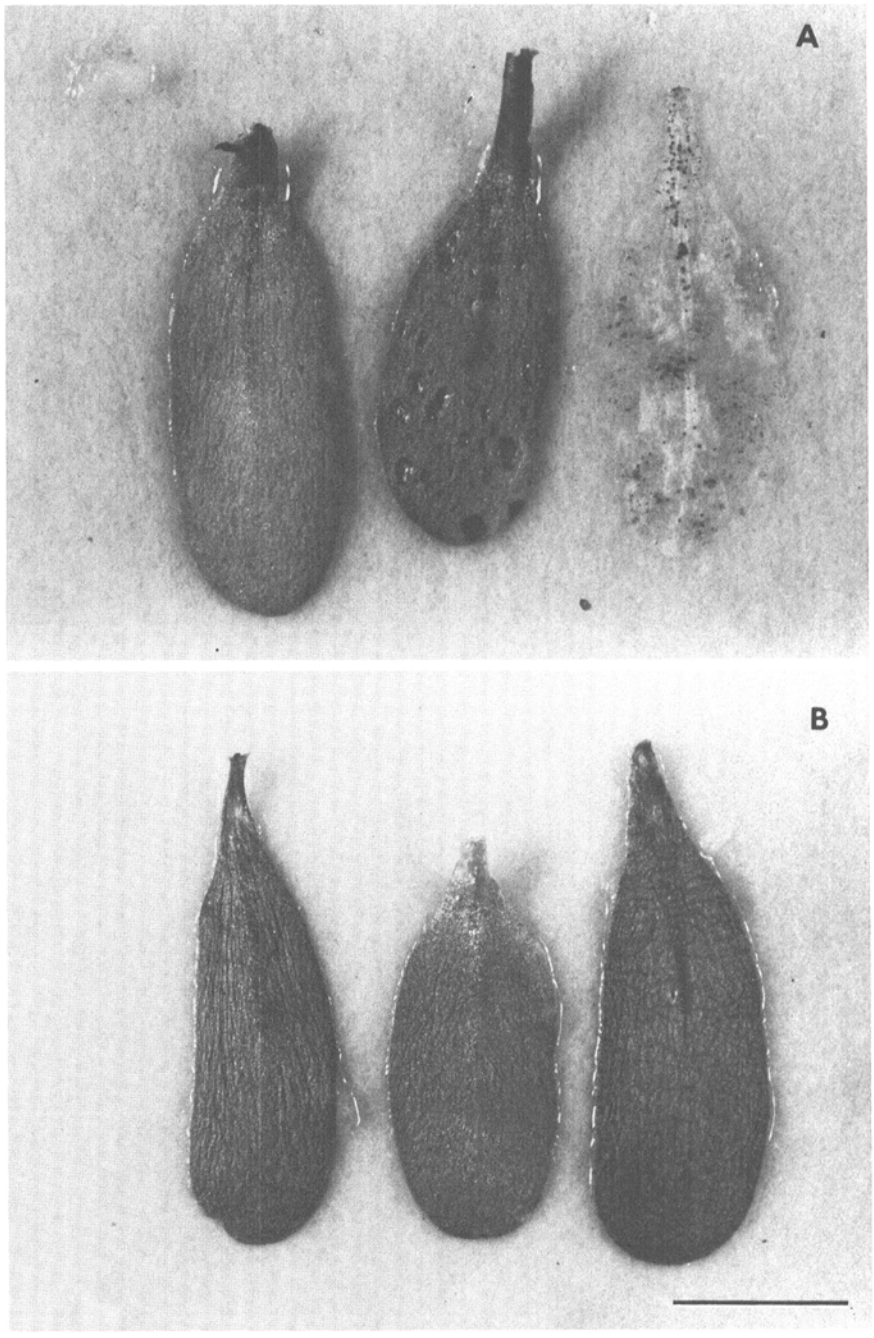

Fig. 1. Excised alfalfa cotyledons showing resistance and susceptibility to *Colletotrichum trifolii* in an in vitro screen. Assay plates were incubated 14 days at 24 C with a 16-hr photoperiod (about 1,000 ft-c). **(A)** Cotyledons were spray-inoculated with *C. trifolii* (5,000 spores per spray). Cotyledon on far left is scored resistant; those in the center and far right are scored susceptible on the basis of the presence of sporulation. **(B)** Cotyledons inoculated with sterile distilled water appear healthy. Scale bar = 5 mm.

Table 2. Effect of inoculum density of *Colletotrichum trifolii* on two cultivars of alfalfa tested with an in vitro cotyledon assay

Inoculum density (no. of spores/spray)	Percent resistance[z]	
	Saranac	Saranac AR
500	10.0 a	55.0 c
2,500	0.8 a	50.0 c
5,000	0.0 a	59.3 c
7,500	0.0 a	56.2 c
10,000	0.0 a	57.7 c
50,000	0.0 a	35.0 b

[z] Each value is the mean of six replicates (plates) with 20 cotyledons per plate. Numbers followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

Table 3. Mean percent resistance of two alfalfa cultivars to *Colletotrichum trifolii* tested by two screening techniques

Screening technique	Mean percent resistance[z]			
	Experiment 1		Experiment 2	
	Saranac	Saranac AR	Saranac	Saranac AR
In vitro (cotyledon)	3.37 a	65.68 b	2.85 a	66.98 b
Greenhouse (seedling)	3.01 a	70.57 b	1.21 a	71.81 b

[z] Each value is the mean of 15 replicates with 10–20 plants per replicate. Numbers followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

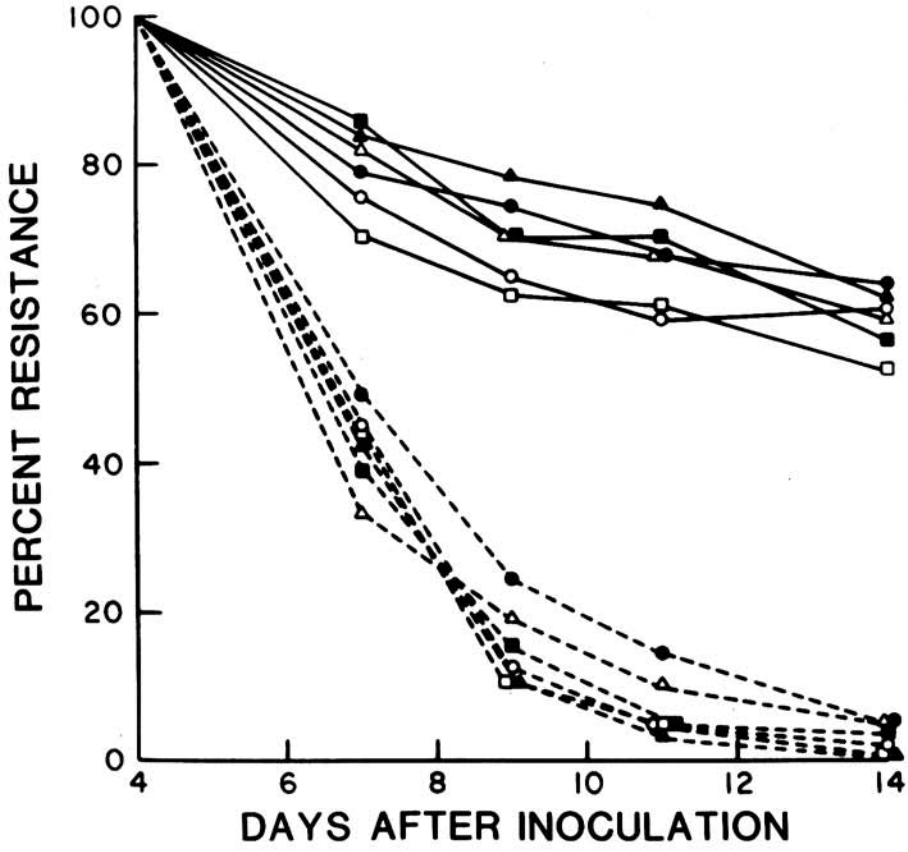

Fig. 2. Percent resistance values over a 14-day incubation period of cotyledons of Saranac (- - -) and Saranac AR (——) inoculated in vitro with different isolates of *Colletotrichum trifolii*: o = F54A, Δ = F55A, □ = F56A, • = F59A, ▲ = F60A, and ■ = combination of all isolates.

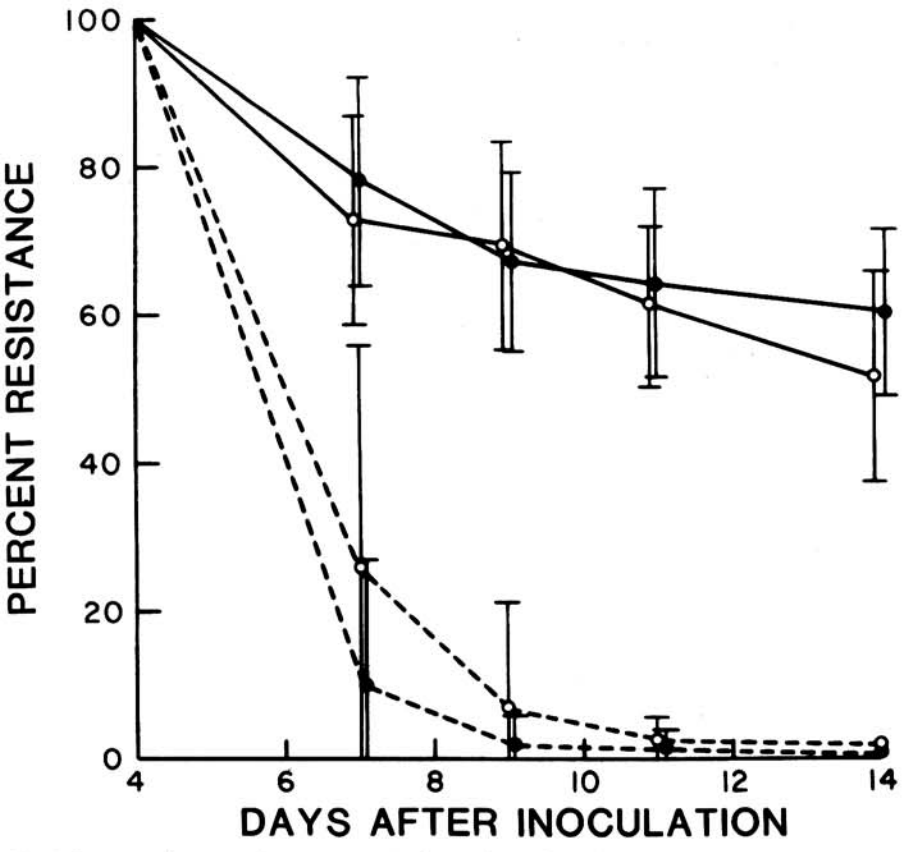

Fig. 3. Percent resistance values over a 14-day incubation period of cotyledons of Saranac (- - -) and Saranac AR (——) inoculated in vitro with *Colletotrichum trifolii* from plants grown in the laboratory (•) and the greenhouse (o).

Raidor. This indicates that this screening technique will accurately assess the level of resistance for a cultivar.

DISCUSSION

The data indicate that this technique is a useful alternative for evaluating the level of resistance to *C. trifolii* present in alfalfa cultivars. The data also show that only a few factors influence the results of this assay. When ideal culture age and incubation conditions are used (8,12), only extremes of inoculum concentration and water levels (J. D. Cucuzza and J. Kao, *unpublished*) drastically influence results. Expression of resistance and evaluation of resistance levels were not significantly influenced by isolates, growth conditions, or sterility; also of no significant influence were age of cotyledons, type of inoculation, or type of plates used (J. D. Cucuzza and J. Kao, *unpublished*).

Although detached plant organs are not commonly used in screening assays, this work indicates that they can be used reliably. Preliminary work with detached trifoliolate leaves indicates a greater sensitivity of these tissues to experimental factors. The storage reserves present in the cotyledon may account for its success under the conditions of this assay.

This technique allows screening of material for resistance or evaluation of new cultivars without introducing *C. trifolii* into a greenhouse or moving plants and soil into a clean laboratory. Present greenhouse screens require 14–30 days of incubation (4,6,7,9); this in vitro assay reduces the time to 10–14 days. In addition, this assay can be conducted year-round and avoid the temperature and light fluctuations that occur in greenhouses. A major advantage is the nondestructive nature of the assay. This allows assessment of resistance for large numbers of seedlings, and susceptible seedlings can be maintained as a whole plant or initiated into tissue culture for genetic and biochemical studies.

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