Assays for Determining Resistance and Susceptibility of Onion Cultivars to the Pink Root Disease

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Research supported by USDA Grant 12-14-100-9986 (34) and Brigham Young University.
Accepted for publication 1 October 1987.

ABSTRACT

Hess, W. M., and Weber, D. J. 1988. Assays for determining resistance and susceptibility of onion cultivars to the pink root disease. Phytopathology 78:115-117.

Biological assays using the pathogen *Pyrenochaeta terrestris*, crude exudates of the fungus, and semipurified preparations of toxic products of the fungal exudates were developed to evaluate resistance and susceptibility of seedlings of various cultivars of onion to the pink root disease. One bioassay involved inoculating onion seedlings in petri dishes with the fungus. Indications of pathogenicity of the fungal isolates were obtained within 7 days. Some indications of resistance of onion cultivars were obtained by this method, but the sensitivity was limited. A bioassay with a crude fungal exudate permitted determination of susceptibility of onion seedlings within 5 to 7 days. The disadvantage of the crude extract assay was that contaminants on seeds of certain cultivars grew rapidly in the carbohydrate material in the crude extract and made interpretation of the results difficult. A bioassay using a semipurified fungal toxin was the most reliable, and evaluation of the susceptibility of onion seedlings was possible in 5 to 7 days without serious problems from contaminants. An efficient procedure for mass production and preparation of the semipurified toxin was developed.

Several weeks to months are required to screen onion cultivars for resistance to the onion pink root fungus *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker & Larson (9,11), and soil treatment procedures take time and are expensive (8). If the amount of time to test resistance could be reduced, onion breeding programs could proceed more rapidly, and considerable time could be saved in developing resistant onion cultivars. Resistance probably could be determined in a few days if toxic products produced by specific isolates of the fungus were used to treat onion seedlings. Not only would one be able to evaluate pink root disease reaction with this method, but resistant seedlings could be selected from the assays and could be planted and grown for further testing. The purpose of these studies was to develop biological assays to evaluate disease reactions with onion cultivars when exposed to toxins of various isolates of *P. terrestris*.

MATERIALS AND METHODS

Pathogenic isolates of *P. terrestris* were obtained by field isolation (3) from onion (*Allium cepa* L.) fields in Utah, Idaho, and Oregon. The cultures of various isolates of the fungus were grown for a few days on standard media, such as potato-dextrose agar, Warcup's medium, or Czapek's medium, to ensure that contaminants were not present. Then they were transferred to screw-top test tubes (about 25 × 150 mm) of sterile loam soil.

The isolates of the pink root fungus were allowed to grow at room temperature until they reached the bottoms of the soil tubes. This took approximately one month. Soil cultures were then placed in cold storage. The viability and pathogenicity of various isolates of the fungus may be maintained for at least a decade by this method if the cultures do not dry out (1,2,10).

Production of crude exudate. Crude fungus exudate was produced from Czapek's medium with powdered alpha-cellulose (Sigma No. C-8002). The medium contained the following: $NaNO_3$ (6.0 g), K_2HPO_4 (2.0 g), KCl (1.0 g), $MgSO_4 \cdot 7H_2O$ (1.0 g), $FeSO_4 \cdot 7H_2O$ (0.02 g), sucrose (60.0 g), cellulose (20.0 g) in 2 L distilled H_2O. The pH was adjusted to 5.0. Cultures of the respective isolates of the fungus were used to inoculate 4-L Erlenmeyer flasks containing 2 L media. After 2 wk, the flasks were placed on reciprocal shakers at 50–60 strokes per minute at 20–22 C, where they were allowed to grow for an additional 4 wk. Each flask was manually shaken 5 days and 10 days after inoculation to disperse the cellulose.

Harvest of crude exudate. When the fungus was grown under these conditions, the hyphal cells surrounded the cellulose in the medium, and filtering the exudate was easy. If the cultures were shaken continuously after inoculation without a 2-wk wait, the fungus did not grow around the cellulose, and the exudate harvest was very slow because filtering was difficult. Large funnels adapted with stainless-steel screens, with a diameter of about 4.5–5.1 cm, upon which sterile glass wool was placed, were used to harvest the exudate. A sterile glass or plastic rod was used to retain the fungus and cellulose mats in the flasks while flasks were inverted to pour off exudate. This process took 3–4 hr because the fungus-cellulose mats retained the exudate. The exudate was then freeze-dried and stored in a freezer.

Preparation of semipurified toxin. The harvested crude fungal exudate was concentrated 10-fold by flash evaporation at 45 C. One liter of concentrated exudate was added to 1,200 ml water-saturated butanol and shaken vigorously, and the solvents were allowed to separate into two phases in a separatory funnel. The top (butanol) layer contained most of the toxic products as determined by seedling assays. The bottom (water) layer was reextracted with 800 ml water-saturated butanol. The butanol fraction was retained and combined with the first butanol fraction. The combined butanol fractions, about 2 L, were washed twice with 1,600 ml distilled water in a separatory funnel. The water fraction contained only small amounts of toxic products, as determined by the seedling assays, and was therefore discarded. The butanol fraction was washed a third time with 1,200 ml distilled water. The mixture was shaken vigorously in a separatory funnel and allowed to stand 2–3 hr or, ideally, overnight. The water fraction was again discarded. The top layer consisted of approximately 1,600 ml butanol with toxic products. The butanol fraction was flash evaporated to dryness at 45 C.

The residue that remained after flash evaporation had an oily consistency and was only partially soluble in water. Most of it was solubilized by addition of 20 ml ethyl ether (not water saturated), followed by 10–15 ml water. This final solution was equivalent to a 500-fold concentration of the culture filtrate. The ether-soluble fraction was stored separately from the water-soluble fraction. If butanol was added to the residue that remained after the removal of the ether- and water-soluble fractions, a butanol fraction could also be removed. All three fractions contained toxic activity. However, as determined by the seedling assays, the ether fraction was more toxic than the other two fractions and was used for onion pink root resistance tests.

Seedling preparation. Seeds were soaked in distilled water for a

TABLE 1. Assay of onion seedlings for sensitivity to crude toxins

Onion cultivars	Seedling growth (mm)[a]					
	25 mg/ml			50 mg/ml		
	Toxin	Control[b]	Percent inhibition	Toxin	Control[b]	Percent inhibition
Susceptible 826B	1.8	3.9	54	0.9	4.7	80
Susceptible 1459B	2.3	3.5	35	0.7	1.7	59
Susceptible W205B[c]	2.56	4.6	45	2.3	6.4	65
Resistant 2935B	2.5	2.8	8	1.0	3.0	40

[a]Average of 100 seedlings for each treatment.
[b]Control was treated with water only.
[c]This was a segregating line.

minimum of 0.5 hr; during this period the water was changed several times. Seeds were vigorously aerated 1 hr, treated with commercial Purex or Clorox diluted 1:4 with water for 10 min, washed with running water for at least 1 hr, and incubated in moist paper towels at 20 C 4–6 days (4,5).

Assay media and procedures. For the seedling assay, 3 ml low-nutrient medium (1 g $MgSO_4$, 3 g $NaNO_3$, 20 g agar in 1-L H_2O) was poured into 3.8-cm-diameter minidishes (Carolina culture dishes 74-0996) (4). Next, 200 μl of the ether-soluble fraction were placed on the agar surface. Then 100 μl of the ether-soluble fraction were added to the sterile filter paper discs (diameter of 26–27 mm). After the ether evaporated, pregerminated seedlings of various cultivars of onion were placed in the minidishes and covered with the toxin-coated filter-paper discs. Sterile distilled water was used to moisten the agar surfaces and filter-paper discs. The control treatments consisted of only sterile water added to the agar surfaces and filter-paper discs.

Because the toxic fraction contained a residue with an oily consistency, standardization involved a relative volume measurement rather than an exact weight measurement. However, in some assays, the residue was weighed and then dissolved in ether to a specific volume.

Seedlings were normally left in the minidishes in a growth chamber at 10,764 lx continuous light 5–7 days at 21 C. The amount of time varied with the susceptibility of the respective onion cultivar to the toxin. Two toxin preparations were studied: one from a pathogenic isolate of the pink root fungus (C-44-1), and WT-1, a pathogenic isolate obtained from Clint Peterson (USDA—University of Wisconsin). The toxin preparation from C-44-1 was more toxic than the toxin preparation from the Wisconsin isolate for most of the cultivars tested.

Resistant seedlings were removed from the minidishes and planted in soil in a greenhouse or growth chamber. Root lengths of seedlings were measured as an index of the toxicity of the crude toxin. For some experiments, it was also necessary to measure stem lengths. It was possible to add enough toxin to prevent root growth of even resistant seedlings. For this reason, the amount of toxin used for assay procedures was an important factor.

Fungus isolate pathogenicity bioassay. A method was used to expose onion roots to pure cultures of several isolates of *P. terrestris* (5). This method worked very well to determine whether isolates of the pink root fungus readily infect roots of various cultivars of onions. It was also useful in determining whether isolates of the fungus had retained pathogenicity in culture.

The method consisted of transferring germinated seedlings to 20 × 100 mm petri dishes containing low-nutrient agar that was previously inoculated with soil cultures of various isolates of the fungus. The surface of the agar was overgrown with the fungus, but the fungus was barely visible because it did not grow well on this medium. If a medium was used that supported good growth of the fungus, the fungus grew on the medium rather than on the roots of the onion plants. Sterile, 9-cm filter-paper discs were placed over the seedlings and were moistened with sterile distilled water. The dishes were placed in a growth chamber at 10,764 lx continuous light at 21 C 5–10 days. Sterile distilled water was added periodically to prevent drying. The roots of some plants grew into the agar and partially escaped the fungus. However, enough roots were infected to determine whether the respective isolates of the fungus were weakly or highly virulent.

TABLE 2. Activity of crude and semipurified toxin from *Pyrenochaeta terrestris*[a]

Sample	Concentration	Percent inhibition[b]
Crude	50 mg/ml	28
Semipurified	50 mg/ml	47
Semipurified	25 mg/ml	27

[a]Average of 100 Red Creole seedlings treated with toxin from isolate C-44-1.
[b]Controls were treated with water only and values were considered to be 0% inhibition.

RESULTS AND DISCUSSION

Pathogenicity assays involving the addition of the fungi directly to the growing seedlings were used. The evidence suggested that pathogenicity could be roughly determined, but the inoculum load was so high that it was not a good measure of resistance of onion seedlings. The relative percent of infection gave an indication of the resistance of the cultivar tested. Nonpathogenic isolates of the fungus did not infect host tissues although pink hyphae were sometimes observed on root surfaces.

Assays involving the crude toxin were undertaken to determine if they could be used to screen for resistance. The assays were replicated 20 times to evaluate variation. Results are shown in Table 1. The assay with crude exudate gave indications of resistance of seedlings of onion cultivars. However, crude exudate contained carbohydrates that supported growth of fungal contaminants. This caused confusion in interpretation of data.

Assays were done with the semipurified toxin and with the crude toxin (Table 2). Fungal contaminants rarely grew when preparations of the semipurified toxin were used. Careful seedling preparation also helped to eliminate the contaminants. The seedling assays for resistance to the toxin correlated positively with field resistance tests in onion fields. With semipurified toxin, comparisons of toxicity of isolates of the fungus and susceptibility of cultivars to the isolates may be studied (Table 3). Thus, time can be saved by using the semipurified toxin for resistance assays.

Therefore, in these investigations, the semipurified toxin was more suitable for pink root resistance determinations than crude exudate. The mixture of toxic components from a single isolate of the fungus or from a culture with mixed isolates makes possible a quick evaluation of seedling susceptibility. Studies need to be conducted to determine whether the use of pyrenocines isolated by Ichihara et al (6,7) and Sparace et al (15), and the pyrenochaetic acids isolated by Sato et al (12–14) would make possible a more precise determination of pink root resistant cultivars of onion.

A major advantage of using purified toxins is that the problem with microbial contaminants would be greatly reduced. On the

TABLE 3. Toxicity of semipurified toxin preparations from two isolates of *Pyrenochaeta terrestris*

Cultivars	Control	Fungal isolates		
		C-44-1	WT-1	LSD (0.05)[a]
Resistant (WA6 B)	8.57	1.84	1.26	0.98
Intermediate (Red Creole)	11.62	1.61	0.92	0.99
Susceptible (4535 B)	5.74	0.59	0.28	2.06
LSD (0.05)[a]	2.38	0.63	0.37	

[a] Average growth of 100 samples (mm) treated with semipurified toxin (15 mg/ml/10 seedlings). LSD values for the 5% level using Fisher's PLSD test.

other hand, this preparation of toxin, when separated by thin-layer chromatography (TLC), contained 10 UV-absorbing bands separated by TLC. Based upon seedling bioassays, the extracts from several of these bands contained toxic materials. The 10 bands yielded 14 peaks with GC-mass spectrometry, but not all of these components were bioassayed. This suggests that other toxic components may still be present that have not been isolated. The semipurified toxin may be more useful in screening for a more general resistance to onion pink root. In addition, there appears to be a significant variation in toxic components obtained from different isolates of the fungus. Hess (3) demonstrated that the virulence of different isolates of *P. terrestris* varied significantly.

To standardize seedling evaluation procedures with any of the procedures mentioned, more information concerning the presence of toxic components produced by various isolates of the fungus is required.

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