CHARACTERISTICS OF THE CHESTNUT BLIGHT FUNGUS ISOLATED FROM SCARLET OAK IN PENNSYLVANIA

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Abstract—More than 100 isolates of the chestnut blight fungus, *Cryphonectria parasitica* (=*Endothia parasitica*), were collected during a survey across Pennsylvania from infected scarlet oaks (*Quercus coccinea*) suffering from basal Cryphonectria cankers. Comparisons were made among isolates, as well as to a standard virulent (relative to American chestnut) and standard hypovirulent isolate in terms of linear growth on agar, lesion area induced in apple fruit, and canker area induced on American chestnut saplings. A ranking test indicated that growth on agar, lesion area induced in apple, and canker size induced on American chestnut were correlated. Most isolates grew on agar, infected apple fruit, and infected chestnut stems in a manner similar to that of the standard virulent isolate. A reservoir of virulent inoculum from scarlet oaks may confound efforts at biological control of chestnut blight on American chestnut. The possibility that a small number of isolates from scarlet oak may be hypovirulent on American chestnut should be investigated because one isolate had characteristics similar to the known hypovirulent isolate.

INTRODUCTION

Cryphonectria parasitica (Murrill) Barr (=*Endothia parasitica* (Murrill) P.J. Anderson & H.W. Anderson), virtually eliminated the American chestnut (*Castanea dentata* (Marsh.) Borkh.) as a forest tree throughout its range in the USA in the early 1900s (Roane and others 1986). Recently, there has been renewed interest in control of chestnut blight on American chestnut through the use of biocontrol (Nuss 1992). However a confounding factor in this biocontrol effort is that the surviving sprouts of American chestnut often grow in the understory of oak forests, and many of companion oak species, including scarlet oak (*Quercus coccinea* Muenchh.), also are susceptible to *C. parasitica* (see review in Torsello and others 1994). Working with oaks in southeastern USA, Nash and Stambaugh (1982) suggested that infected oaks could serve as reservoirs of virulent *C. parasitica* inoculum, especially in forest stands where American chestnut has been eliminated They also indicated that the virulence of some of the southeastern oak isolates, when placed into American chestnut, was equal to or greater than those isolates originally obtained from American chestnut (Nash and Stambaugh 1987).

In addition to harboring these virulent isolates of the chestnut blight fungus, scarlet oak may also contain less virulent, or hypovirulent, strains of the fungus. In fact, some biocontrol efforts in controlling chestnut blight in American chestnut are based on the use of this hypovirulence (Nuss 1992). Although hypovirulent isolates of *C. parasitica* have not been reported from hosts other than American chestnut, infected oaks may represent a potential, unstudied reservoir of hypovirulence for use in this biocontrol effort. Determination of isolate virulence with respect to American chestnut involves inoculation and evaluation of canker development on chestnut stems in the field (Elliston 1982, 1985; Scibilia and Shain 1989). However, such field trials are time consuming, and the limited number of uninfected chestnut stems present in some forest stands may not allow studies involving large numbers of isolates. Therefore, Elliston (1982, 1985) suggested inoculation of apple fruit as a rapid and efficient means to initially screen large numbers of isolates of *C. parasitica* to estimate virulence. Fulbright (1984) reported that the rate of colonization of "Granny Smith" apple fruit infected by *C. parasitica* isolates obtained from American chestnut might be used to estimate relative virulence of the same isolates in chestnut. In addition, Bedker (1989) suggested that linear growth rate in culture might be used to estimate the virulence of *C. parasitica* isolates in chestnut.

Linear growth in culture and ability to colonize apple fruit, in conjunction with chestnut stem inoculations to evaluate virulence, have not been examined for isolates of *C. parasitica* from scarlet oak. If successful, estimation of potential virulence of isolates based on linear growth in culture and/or apple colonization would allow a quick identification of source material for further studies dealing with virulence of *C. parasitica*. Also, inoculation of American chestnut stems under field conditions may yield insights into the possible role of inoculum from oaks on biological control of chestnut blight.

The objective of this study was to compare isolates of *C. parasitica* from scarlet oak growing in Pennsylvania, in terms of linear growth in culture, lesion size produced in apple fruit, and canker size induced on American chestnut stems. Comparisons were also made to known virulent or hypovirulent isolates.

METHODS

Linear Growth in Culture

During a survey across Pennsylvania, we collected 102 isolates of *C. parasitica* from cankered scarlet oak (SO);

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detailed methods regarding isolation and culturing have been presented (Torsello and others 1994). Initial isolations were cultured on acidified Difco potato dextrose agar (aPDA, 1 ml of 85 percent lactic acid per liter of potato dextrose agar). Subsequent cultures were maintained on acidified PDA containing methionine (100 mg/l) and biotin (1 mg/l) (PDAmb) (Anagnostakis and Aylor 1984). Occasionally, more than one SO isolate was obtained from a single canker. Since several vegetative compatibility types can occur in a single canker (Nash and Stambaugh 1989), each isolate was treated as a distinct entity. The linear growth of the 102 SO isolates was compared to the linear growth of EP155 and EP713, two *C. parasitica* isolates of known virulence. EP155 is a "standard" (Anagnostakis 1992) virulent isolate, American Type Culture Collection (ATCC) #38755, and has a normal phenotype. EP713 is a slow-growing, hypovirulent isolate (ATCC #52571) (Anagnostakis 1992). EP155 was obtained from W. MacDonald, West Virginia University, and EP713 was obtained from S. Anagnostakis of The Connecticut Agricultural Experiment Station.

One 7-mm diameter plug of PDAmb with mycelium was removed from each 7-day-old SO isolate, as well as EP155 and EP713, and placed on PDAmb against the wall of a petri plate. Four sets of each isolate were grown upside down in petri plates to minimize moisture accumulation on the agar surface. Plates were maintained in darkness at 21°C on four shelves in a controlled environment room utilizing a randomized block design with two replications. Shelves located perpendicular to a possible vertical temperature gradient in the room were considered as blocks. Each block consisted of a complete set of 102 SO isolates, EP155, EP713, and a control. After 7 days, the morphology of each colony was described and the growth of each isolate measured by taking two linear measurements per plate, from the edge of the plug to the distal edge of the colony, and averaged.

The 102 SO isolates, EP155 and EP713 were ranked in order of linear growth produced after 7 days (Minitab 1991). Growth data also were analyzed using ANOVA and Tukey's Honestly Significant Difference (HSD) multiple comparison procedure (p=0.05) to determine if significant differences in growth occurred among isolates (SAS 1985).

Apple Inoculations

One isolate from the linear growth study failed to grow. Therefore, 101 SO isolates, EP155, and EP713, plus a sterile PDAmb plug as a control were used to inoculate apple fruit. Fruit of the apple cultivar "Granny Smith" were sorted for uniformity, washed with sterile distilled water and detergent, wiped with 65 percent ethanol, and placed in a laminar flow hood. Each fruit was labeled with a waterproof marker and inoculated with three isolates. Each apple was inoculated with EP155 as a standard; the remaining two inoculations per apple were either SO isolates, EP713, or the control. A 7-mm diameter plug was removed from each fruit using a sterile cork borer, and a 7-mm plug of PDAmb containing the isolate, or sterile PDAmb agar as a control, was placed into the hole. Non-absorbent cotton was placed over the plug and the apple was wrapped with a strip of parafilm to minimize evaporation. Apples were placed on five vertically arranged shelves in a randomized complete block design. Shelves were considered as blocks against a possible vertical temperature gradient. Inoculated apples were incubated in the dark in a controlled-environment room set at 22°C. Temperature was recorded daily on shelves using glass thermometers.

Isolates, except for EP155, were randomly selected for inoculation of each apple within a block. Inoculated fruits were then randomized with respect to shelf position. Lesion length (mm) and width (mm) on each fruit were measured on the 12th day after inoculation. The experiment was terminated after 12 days since isolate interactions within individual fruits became a possibility as the lesions enlarged. Lesion area was determined and standardized by dividing the lesion area induced by each test isolate by the lesion area induced by the standard EP155 on the same apple.

The 101 SO isolates, as well as EP155 and EP713, were ranked in terms of lesion area induced (Minitab 1991). Data also was subjected to General Linear Model (GLM) analysis and Tukey's HSD mean comparison test (p=0.05) was used to test for differences in mean lesion areas induced by the various isolates (SAS 1985).

Chestnut Inoculations

Eighteen American chestnut sprouts growing in a 10-year-old clearcut in the Savage River State Forest, New Germany, Maryland were selected for inoculation. Sprouts selected did not have *C. parasitica* cankers within the proposed inoculation height on the main stem (0.5m to 3.0m), nor any other obvious large cankers on the stem. Due to the limited number of suitable stems available, all isolates could not be tested. Five of the *C. parasitica* isolates which induced the largest lesions on apple fruit(SO isolates 9, 31, 57, 77, and 86), and five of the isolates which induced the smallest lesions (SO isolates 12, 17, 39b, 69 and 99) were selected, along with the standard virulent EP155, hypovirulent EP713, and a PDAmb agar control.

On May 17, 1991, four inoculation wounds per stem were made by removing circular areas of bark with a sterile, 7-mm diameter core borer. Plugs of inoculum from 7-day-old cultures of *C. parasitica* on PDAmb, or sterile agar plugs as controls, were placed in the wound (mycelium towards the pith) and covered with parafilm. Each chestnut sprout was inoculated with the standard virulent isolate EP155. The remaining three wounds on each stem received the SO isolates, the hypovirulent EP713, or the control in a random distribution. Three repetitions of each isolate, other than EP155, were used. Inoculation wounds were not placed directly beneath one another on the same stem to minimize the possibility of conidia spread by stemflow from one inoculation point to another. Inoculation points were spaced at maximum allowable distances to minimize over-lapping canker growth between or among isolates.

Length (mm) and width (mm) of each canker was measured on August 29, 1991, 104 days after inoculation.

Field observations during canker development indicated that this time period was sufficient to assure that cankers were well established, yet not so large as to girdle the stem or overlap. The linear length and width measurements were converted to area prior to analysis. Since stems were variable in size and shape, lesion area was standardized by dividing the area induced by each isolate by the area induced by EP155 on the same stem, and ranked (Minitab 1991). The ranking was compared to the rankings for linear growth in culture, as well as the ranking for lesion size induced in apple fruit, and tested using Spearman's rank correlation (Minitab 1991). Data also were subjected to GLM analysis and Tukey's HSD mean separation test (p=0.05) (SAS 1985).

RESULTS

Linear Growth in Culture

There was no significant difference in linear growth among blocks (shelves in the incubator), but mean growth between the two replications was significantly different. However, in replication 2, growth of most isolates was poor and erratic, apparently due to laboratory techniques. Therefore, only data from replication one is presented in this paper (see Torsello (1992) for data from replication two).

Few significant differences in linear growth were observed among isolates; complete datasets for all 102 SO isolates are not presented, but have been reported elsewhere (Torsello 1992). Only isolate 68a of the 102 SO isolates grew significantly greater than that of the standard virulent strain EP155. Isolates 81 and 99, as well as the hypovirulent EP713, grew significantly less than EP155. The linear growth of SO isolate 99 and the hypovirulent EP713 were similar and were significantly less than all other isolates. There were no significant differences in growth among the remaining SO isolates.

The general culture morphology of all isolates, except for SO isolate 99, was similar to that of EP155, the standard virulent isolate. The morphology of isolate 99 was similar to that of the known hypovirulent EP713.

Lesion Induction in Apple

Monitoring with thermometers within the controlled environment room revealed no temperature gradient. However, apples on block 2 (shelf 2) had a slightly, but significantly, greater mean lesion area as compared to block 4 (shelf 4). Other comparisons among blocks were non-significant. Scarlet oak isolate 99 induced significantly smaller lesions on the fruit compared to all other isolates, including the standard hypovirulent EP713 and the standard virulent isolate EP155. Only SO isolates 22 and 47 induced significantly larger lesions than the hypovirulent EP713, but the lesions were not significantly different in size from those induced by EP155 (Torsello 1992). Other isolates induced lesions on apples not significantly different from those induced by EP155. The sterile agar plug resulted in minimal browning around each inoculation wound.

Canker Induction in Chestnut

Data from one stem were eliminated due to failure of EP155 to induce lesions, which precluded standardization. Considerable variation in canker size was noted, even among repetitions of the same isolate. Six isolates induced lesions larger than the standard EP155, and five isolates induced cankers smaller than EP155, but differences in lesion area among replications, individual stems, or isolates were not significant (data not presented). Isolate 99 induced the smallest lesion of all isolates. The sterile agar plug resulted in minimal, but measurable, browning around some inoculation wounds.

Ranking of Isolates

The relative ranking, standardized with reference to EP155, of canker size induced on American chestnut by 10 isolates and hypovirulent strain EP713 is shown in Table 1. Rankings from the apple fruit inoculations were very similar to the rankings derived from the chestnut stem inoculations, with a significant (p=.01) Spearman's rank correlation coefficient of 0.874. Likewise, the rankings from the first replication of the linear growth study correlated significantly (r=0.877, p=.01) with the rankings from the chestnut inoculations. However, rankings from the second replication of the linear growth study were not correlated (r =-.009, p=non-sig.) with rankings derived from the chestnut inoculations.

DISCUSSION

In terms of linear growth on agar, there were few differences among the 102 SO isolates of *C. parasitica* (See Torsello 1992 for complete dataset). Only scarlet oak isolate 99 (SO99) and the known hypovirulent EP713 (Anagnostakis 1992) grew significantly less than the standard virulent EP155 in both replications. The general culture morphology of all isolates except SO99 was similar to the phenotype of EP155 in both replications. SO99 exhibited submerged hyphae, reduced fruiting, and a mycelial morphology similar to that of some hypovirulent strains (Anagnostakis 1990). Based on linear growth in culture, we conclude that most of the SO isolates to be more similar to the virulent than to the hypovirulent strains, with the exception of SO99.

With regard to apple fruit, most of the 102 SO isolates induced lesions similar in size to those caused by the standard virulent isolate EP155. However, SO99 induced significantly smaller lesions on the fruit compared to all other isolates, including the standard hypovirulent EP713 and the standard virulent isolate EP155, again indicating characteristics of possible hypovirulence (Fulbright, 1984).

On the stems of American chestnut, considerable variation in canker size was noted, even among repetitions of the same isolate. Six isolates induced lesions larger than the standard EP155, and five isolates induced cankers smaller than EP155 (Table 1), but differences in lesion area among replications, individual stems, or isolates were not significant (Torsello, 1992). The lack of significance in the chestnut stem canker data is attributed in part to a severe drought that occurred during canker development, arresting growth of some inoculated saplings and causing mortality

Table 1—Relative ranking of growth of the 12 *C. parasitica* isolates which were common to all studies, in terms of canker size (ratio) produced on American chestnut, lesion size (ratio) induced in apple fruit, and linear growth (mm) in culture

Chestnut stem			Apple fruit		Linear growth	
ID	Area	Rank	Area	Rank	mm	Rank
99	0.450[a]	1	0.216[b]	1	7.00[c]	1
69	0.508	2	0.716	6	39.25	7
713[d]	0.523	3	0.608	2.5[e]	14.00	2
12	0.585	4	0.618	4	38.75	5
17	0.642	5	0.608	2.5	37.50	3
155[d]	1.000	6	1.000	7	39.00	6
39b	1.126	7	0.638	5	38.00	4
77	1.399	8	1.236	10.5	41.50	8.5
9	1.702	9	1.222	9	44.50	12
86	5.078	10	1.236	10.5	41.50	8.5
31	5.225	11	1.198	8	43.75	11
57	6.252	12	1.300	12	43.00	10
Correlation coefficient:			0.874[f]		0.877	

[a] Ratio of (canker area induced by each isolate) / (canker area induced on the same stem by EP155) 104 days after inoculation.
[b] Ratio of (lesion area induced by each isolate) / (lesion area induced on the same apple fruit by EP155) 12 days after inoculation.
[c] Linear growth (mm) after 7 days on PDAmb.
[d] 155 is the standard virulent isolate (EP155) and 713 is a known hypovirulent isolate (EP713).
[e] The same number (ending in .5) appearing within a column indicates equal ranking.
[f] Spearman's rank correlation coefficient comparing the relative ranking of respective columns to the results of the American chestnut inoculations.

of others, thus reducing the sample size and introducing variability. This variability confounded the critical comparison between the stem inoculation results with the linear growth and/or apple inoculation results, since stem canker production is the ultimate test for hypovirulence. However, it is important to note that, of all the isolates studied, SO99 induced the smallest lesion on American chestnut.

The relative rankings (Table 1) also reveal that the virulence ranking based on apple fruit inoculations were very similar to the rankings derived from the chestnut stem inoculations. Also, the rankings from the first replication of the linear growth study correlated significantly with the rankings from the chestnut inoculations. However, rankings from the second replication of the linear growth study were erratic, and not correlated with rankings derived from the chestnut inoculations, thus confounding any comparisons of our results with those of Bedker (1989). The rankings do support the use of inoculation of apple fruit as a possible means to initially screen large numbers of isolates of *C. parasitica* to estimate virulence (Elliston (1982, 1985; Fulbright, 1984). However, because of the results of replication 2 of the linear growth study, these results do not necessarily support those of Bedker (1989) who suggested that linear growth rate in culture might be used to estimate the virulence of *C. parasitica* isolates in chestnut.

Isolate SO99 should be investigated for the presence of viruses conferring hypovirulence. Hypovirulent isolates of *C. parasitica* have not been reported from hosts other than chestnut. However, results from this study indicate that only a small percentage of the isolates (perhaps 1 percent) from scarlet oak have characteristics of those known to be hypovirulent. If hypovirulence is present within *C. parasitica* on scarlet oaks, even in a small percentage, this population should be considered when formulating biocontrol efforts of chestnut blight on American chestnut involving use of hypovirulence.

However, most of the isolates collected from scarlet oaks across Pennsylvania had characteristics similar to the known virulent isolate, EP155. We concur with Nash and Stambaugh (1982), who found that cankered oaks in the southeastern USA may serve as important reservoirs of virulent *C. parasitica* inoculum, and that virulence (in

American chestnut) of some oak isolates is equal to or greater than that of isolates originally obtained from American chestnut.

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