Characterization of Induced Resistance to Anthracnose in Alfalfa by Races, Isolates, and Species of *Colletotrichum*

Nichole R. O'Neill, James H. Elgin, Jr., and C. Jacyn Baker

First author: Germplasm Quality and Enhancement Laboratory; second author: deputy area director, Plant Sciences Institute; and third author: Microbiology and Plant Pathology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of the products that may also be suitable. This article reports the results of research only.

The statistical assistance of R. P. O'Neill and M. J. Camp is gratefully acknowledged. We wish to thank S. Stanton for technical assistance.

Accepted for publication 11 January 1989 (submitted for electronic processing).

ABSTRACT

O'Neill, N. R., Elgin, J. H., Jr., and Baker, C. J. 1989. Characterization of induced resistance to anthracnose in alfalfa by races, isolates, and species of *Colletotrichum*. Phytopathology 79:750-756.

The phenomenon of induced resistance and its biochemical and physiological basis is being investigated in the anthracnose disease of alfalfa (*Medicago sativa*). By prior inoculation with race 1 of *Colletotrichum trifolii*, race 1-resistant, race 2-susceptible cultivars such as Arc can be protected against subsequent infection by race 2. In this study we investigated the effectiveness of race 1 inoculum concentrations on induced resistance, the effectiveness of protection against high race 2 disease pressure, and the association of pathogen virulence with the capacity to induce resistance. Protection of Arc seedlings against race 2 was highly correlated with increasing spore concentrations of race 1. Arc has no specific genes for resistance to race 2, and a gene for resistance to race 1 present in 65 to 75% of the plants of this cultivar permits a similar level of survival in inoculated seedlings. An inducing concentration of 2×10^6 race 1 spores/ml followed by challenge inoculation 24 hr later with race 2 permitted 55% seedling survival, whereas all unprotected seedlings were killed. Race 1 protection was not diminished by high disease pressure from concentrations of race 2 inoculum of up to 4×10^6 spores/ml. Relative virulence to resistant host genotypes varied significantly among isolates of race 1 and was associated with their ability to induce protection in Arc against race 2. Of other species of *Colletotrichum* tested, an isolate of *C. malvarum* and one of *C. gloeosporioides* induced significant protection against anthracnose.

Additional keywords: elicitors, immunization, induced protection, phytoalexins.

Anthracnose, caused by *Colletotrichum trifolii* Bain & Essary, is an important disease of alfalfa (*Medicago sativa* L.), limiting growth and forage yield and decreasing plant vigor (1,6,7). The disease has been controlled primarily through the use of resistant cultivars developed with multiple cycles of phenotypic selection for resistance (1,9). Earlier work (3) showed that *C. trifolii* gains ingress in both resistant and susceptible cultivars by direct penetration of the epidermis via a penetration peg from an appressorium. Porto et al (27) also found that spore germination and appressoria formation were not affected by host genotype. The fungus spreads rapidly throughout susceptible hosts, resulting in oval to diamond-shaped lesions on the alfalfa stem. Acervuli produce spores on the stem surface and supply secondary inoculum. Crown tissue may become infected, resulting in plant death or predisposition to winter injury (15). In resistant hosts, the fungus penetrates the epidermis but fails to form infection hyphae and ramify throughout the tissue (3). These results suggest that incompatibility is not associated with the failure of conidia to germinate or to form appressoria with penetration pegs, and that resistance is expressed near the time of epidermal cell wall penetration.

In 1978, *C. trifolii* was found to exist as two physiological races, each exhibiting distinct virulence and avirulence interactions with different cultivars of alfalfa (9,23,35). This specificity can be obtained routinely and reproducibly by inoculating seedlings or mature stems with spores (9,24). Resistance to race 1 and race 2 is conditioned independently by a different, single, dominant gene, designated An_1 and An_2, respectively. These genes are inherited tetrasomically (10) in cultivar Arc (RS phenotype, indicating the race 1 resistance gene and lack of the race 2 resistance gene, respectively) and cultivar Saranac AR (RR phenotype, indicating the presence of the race 2 resistance gene, which confers resistance to both race 1 and race 2). The An_1 race 1 resistance gene in Arc and Saranac AR are the same (8). Cultivar Saranac (SS phenotype) is susceptible to both races.

Induced resistance recently has been demonstrated in alfalfa plants either spray or needle inoculated with race 1 of *C. trifolii* (24). In this study certain alfalfa clones and most seedlings from Arc lacking specific resistance to race 2 of *C. trifolii* were protected

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1989.

against race 2 by prior inoculation with race 1 of the same organism. Ostazeski and Elgin (25) determined that induced resistance was genetically based, demonstrating that genes for resistance to race 1 were a requirement for protection against race 2. The induction of systemic and localized resistance, by which host plants are protected against pathogens by prior inoculation with the same or other pathogens, has been demonstrated in several fungal host-pathogen systems, including extensive studies of interactions with cucumber (*Cucumis sativus* L.), French bean (*Phaseolus vulgaris* L.), and species of *Colletotrichum* (2,11,14, 18,19,28,31). Research efforts have focused principally on describing this phenomenon, and more recently mechanisms of induced resistance have been studied (17,30). In some cases, phytoalexin accumulation in response to inducer fungi has been associated with protection (13,20,32).

The present study was initiated to complement other ongoing research focused on developing a model system for elucidating the biochemical, genetic, and molecular basis for virulence and resistance as well as induced resistance in the *M. sativa-C. trifolii* disease interaction. This paper provides evidence for the effectiveness of induced resistance by characterizing the effect of isolates, virulence, inoculum concentration, and other *Colletotrichum* species. Isolates of race 1 and race 2 of *C. trifolii* were examined to determine whether individual isolates vary in virulence to alfalfa cultivars with specific genes for resistance to these races. The relative virulence of race 1 isolates was compared with their capacity to induce race 2 resistance in an RS phenotype. We determined the effectiveness of race 1 inoculum concentrations in inducing race 2 resistance in alfalfa and the effect of various race 2 spore concentrations in challenging protected alfalfa. Species of *Colletotrichum* other than *C. trifolii* also were tested for their ability to induce resistance to anthracnose. A preliminary report has been published (22).

MATERIALS AND METHODS

Fungal isolates. Cultures of field isolates of *C. trifolii* race 1 (isolates 23R1, Vertbeck, HN82, MDMK, TpRe1, 27, Re1, 5-3, 3-5, 2sp2, Classic, and 271) and race 2 (isolates S1-1, SB-1, H5-3, S2-4, SB-2, H4-2, H5-1, S1-3, FVT-4, S2-3, H6-1, and So.farm) were obtained from different geographic locations in Maryland and North Carolina (Stanley Ostazeski, *personal communication*). Cultures were derived from single spores and stored in sterile soil at 4 C. Isolates of *C. coccodes* (Wallr.) Hughes (from tomato), *C. dematium* (Pers. ex Fr.) (from tomato), *C. gloeosporioides* (Penz.) Sacc. (from tomato), *C. atramentarium* (Berk. & Br.) Taubenh. (from potato), and *C. destructivum* O'Gara (from crown vetch) were provided by Thomas Barksdale, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD. *C. gloeosporioides* isolates 47RR and 57RR were isolated from diseased alfalfa growing in our greenhouse. An isolate of *C. malvarum* (A. Braun et Caspari) Southworth was provided by David TeBeest, University of Arkansas, Fayetteville. Isolates were used individually or inoculum was combined as a mixture, as indicated in each experiment. In preliminary experiments, isolates of both race 1 and 2 exhibited differences in cultural morphology and growth rates on solid media, and all exhibited optimum germination (greater than 80%) at 25 C. Race identity of all isolates of *C. trifolii* was confirmed in mature plants of six clones each of SS, RS, and RR alfalfa genotypes by stem injection inoculation with spore suspensions, as described by Ostazeski and Elgin (24).

Inoculum preparation. Inoculum was prepared by culturing isolates on half-strength oatmeal agar (36 g of Difco oatmeal agar, 1 L of distilled water, 7.5 g of agar) in petri dishes for 7 days at 21 C under 12-hr fluorescent light. Spore suspensions were prepared by scraping spores from the agar plates flooded with sterile distilled water containing two drops of Tween 20/L. The suspension was filtered through two layers of cheesecloth to remove mycelial fragments. Spore concentrations for all inoculations were determined by adjusting dilutions to fit spectrophotometer absorbances (720 nm) to a standard curve prepared from hemacytometer counts. Spore concentration was adjusted to 2×10^6 spores/ml unless indicated otherwise. In preliminary experiments, spore germination of *C. trifolii* often was less than 20% when inoculum was prepared by suspending spores in nonsterilized tap or deionized water. In distilled or sterilized water, spore germination and appressorium formation in all isolates was greater than 90% and was not reduced by the addition of two drops of Tween 20/L (N. R. O'Neill, *unpublished*). To account for possible isolate variations in virulence due to differences in spore germination, inoculum was checked for percent spore germination in each experiment at the time of inoculation. Plates of half-strength oatmeal agar were sprayed with each inoculum preparation and incubated in the mist chamber at 23 C for 24 hr. The plate was removed, and 200 randomly selected spores on a disk from the plate were examined microscopically for germination. In earlier experiments, no differences were found in spore germination and appressorium formation on seedling stems of resistant or susceptible clones inoculated with either race (3).

Growth of plants. The seed used in these experiments was from the same seed lots used in standard tests to characterize anthracnose resistance in alfalfa cultivars Arc, Saranac, and Saranac AR, and these cultivars served to differentiate the two races (26). Arc seed was obtained from a foundation seed lot. Because alfalfa is an autotetraploid and is cross pollinated, it is difficult to completely remove recessive genes from the population. Therefore only a portion of the seedlings in the population carries a gene or genes for resistance to anthracnose. Previous anthracnose resistance evaluations with Arc (RS phenotype) showed approximately 65–75% of seedlings with resistance to race 1 and 0% with resistance to race 2 of *C. trifolii* (9,26). Saranac (SS phenotype) and Saranac AR (RR phenotype) were obtained from certified seed lots. Saranac is uniformly susceptible to both races, and Saranac AR is approximately 59% resistant to race 1 and 53% resistant to race 2 (9). Preparation of plant material and inoculation procedure were similar in each experiment. Fifty seeds of a cultivar were planted in replicated, 10-cm plastic pots containing pasteurized soil and placed in a 3×5 m growth room. Temperature was maintained at 25 ± 1 C with a 16-hr photoperiod at a photosynthetic photon flux of 200 $\mu E\ sec^{-1}\ m^{-2}$ measured at plant height.

Spray inoculation of seedlings. Ten days after planting, the number of seedlings emerged was counted for each pot. At 14 days after planting, pots were removed from the growth room and sprayed to run-off (approximately 3 ml/pot) with the appropriate spore concentration of the isolate or isolate mixture using a Binks model 15 spray gun (Binks Manufacturing Co., Chicago, IL) at 15 psi. Pots were placed in a dark mist chamber at 22 C, and the chamber was spray misted with distilled water for 1 min/hr to maintain 100% relative humidity. Pots were removed after 48 hr and returned to the growth room for further disease development. In cases when race 1 was being tested for ability to induce protection, pots were removed after 24 hr, inoculated with race 2, and returned to the mist chamber for an additional 24 hr.

Disease evaluation. Resistance or virulence was assessed 7 days after inoculation as a percent of the stand count taken 10 days after seeding. Each seedling was evaluated and scored as resistant (no lesions or only small, nongirdling lesions) or susceptible (seedling killed or with large, coalescing or sporulating lesions). Relative virulence was expressed as percent seedlings surviving in each isolate-cultivar combination. Induced resistance was expressed as percent seedlings surviving inoculation with race 1 followed by challenge inoculation with race 2. All experiments were repeated at least twice. To stabilize the variance in the percentage data, we used the arc sine square root transformation of the percentage survival means. Transformed means were subjected to analysis of variance and separated by the Duncan Waller method (29). In experiments with other species of *Colletotrichum*, leaves were taken from Arc seedlings 24 and 72 hr after inoculation, cleared in chloral hydrate (3 g/ml of distilled water), stained for 1–3 hr in 0.05% Trypan blue, and examined microscopically for percent spore germination, appressoria, and hyphal development. At least 50 spores were counted from each isolate.

RESULTS

Virulence of race 1 and 2 isolates of *C. trifolii* on alfalfa cultivars. The variability in virulence of nine isolates of race 1 and 12 isolates of race 2 was determined on Saranac, Arc, and Saranac AR alfalfa cultivars. Treatments consisted of three replications of each isolate-cultivar combination and three uninoculated controls. Isolate replications within each race-cultivar combination were arranged in three blocks in the mist and growth chambers, and each race-cultivar combination was analyzed separately. Germination of spores in the inoculum of all isolates was greater than 75% in vitro, and anthracnose developed in all inoculated treatments. The uninoculated controls included in this experiment remained free of anthracnose and were excluded from the statistical analysis. The race designation of all isolates was confirmed. Race 1 isolates killed Saranac but not Arc (RS phenotype), and race 2 killed Saranac and Arc, but not Saranac AR (RR phenotype) (Table 1). Isolate SB-1 was highly virulent to Saranac AR. Analysis of variance indicated significant differences in virulence among race 1 isolates on Arc and Saranac AR. The range in percent resistance in Arc and Saranac AR among race 1 isolates was 52.3–79.2% (mean 67.9%) and 44.4–86.8% (mean 61.6%), respectively. Isolates of race 2 were even more variable in virulence, as indicated by a range of 11.7–68.0% (mean 39.3%) survival of Saranac AR seedlings.

Relative ability of race 1 isolates to induce protection in Arc and relationship to virulence. The relative ability of race 1 isolates to induce protection in Arc seedlings was examined after challenge inoculation with race 2. Two-week-old seedlings of Saranac and Arc were inoculated with each isolate of race 1 at a spore concentration of 2×10^6 spores/ml of distilled water and incubated 24 hr. Half of the Arc seedlings then were challenged by inoculation with spores from a mixture of race 2 isolates at 2×10^6 spores/ml of distilled water. Pots were incubated in the mist chamber for an additional 24 hr and then placed in the growth room for disease development and evaluation. An analysis of variance indicated no significant source of error between the two runs of the experiment with respect to the Arc treatments, but there were differences between experiments in the reaction of Saranac due to somewhat more severe disease development in the first experiment. Data analysis of the first experiment is shown in Table 2.

In vitro spore germination from inoculum from all isolates and isolate mixtures was greater than 75% and was considered adequate for disease development. Few Saranac seedlings survived inoculation with any race 1 isolate and only 3.4% of Arc seedlings survived inoculation with race 2 alone (Table 2). Virulence of race 1 isolates varied significantly ($P = 0.05$) on Arc, ranging from 58.2 to 79.6% seedling survival. The ability of race 1 isolates to protect Arc against subsequent inoculation with race 2 also varied significantly, ranging from 15.3 to 55.2% seedling survival.

Data from both runs of the experiment were plotted to show the relationship between relative virulence of isolates of race 1 on Arc and the ability of these isolates to induce resistance in Arc to race 2 (Fig. 1). Although because of destructive testing these data cannot be analyzed according to the strict definition of a regression analysis model, the plot indicates a possible linear association between virulence and protection. The capacity of an isolate to induce higher levels of resistance (by allowing a greater percent of challenged seedlings to survive challenge inoculation) appears to be associated with higher virulence of these isolates. Conversely, isolates exhibiting low virulence induce lower levels of protection.

Effect of race 1 and race 2 inoculum concentrations on resistance induced in Arc alfalfa. In this experiment, we assessed the effect of inoculum concentration of race 1 (inducer) on subsequent protection in alfalfa from challenge inoculation with race 2. Increasing inoculum concentrations of race 2 also were used to determine whether the resistance induced by race 1 could be overcome by high inoculum pressure. Spore suspensions from several isolates within each race were combined and used as sources of inoculum. Differences between individual isolates were not determined.

Pots of Arc alfalfa were inoculated with race 1 spore concentrations of 0.0, 0.0625, 0.125, 0.25, 0.50, 1.0, 2.0, or 4.0 $\times 10^6$ spores/ml and put in the mist chamber for 24 hr. Pots were

TABLE 1. Evaluation of race 1 and race 2 isolates of *Colletotrichum trifolii* for virulence to alfalfa cultivars

Isolate of *C. trifolii*	Virulence (percent survival)[x]		
	Saranac (SS)[y]	ARC (RS)	Saranac AR (RR)
Race 1			
Rel	16.1 a[z]	79.2 a	86.8 a
3-5	5.6 b	75.6 ab	47.5 bc
27	0.7 c	72.4 ab	60.0 bc
Classic	0.0 c	69.0 abc	44.4 c
23R1	1.3 bc	68.5 abc	62.4 bc
271	3.4 bc	66.4 abc	69.5 abc
5-3	1.3 bc	65.0 abc	72.7 ab
2sp2	0.0 c	62.6 bc	50.0 bc
TpRe 1	0.0 c	52.3 c	61.2 bc
Mean		67.9	61.6
Race 2			
H5-1	5.1 a	4.4 a	68.0 a
H6-1	0.0 b	1.7 ab	53.5 ab
S1-1	0.6 b	0.8 b	45.0 bc
H5-3	0.0 b	0.0 b	41.7 bc
SB-2	1.9 ab	2.0 ab	41.0 bc
S1-3	0.8 b	0.0 b	40.0 bc
So. farm	0.0 b	0.0 b	39.6 bc
H4-2	4.5 a	1.4 a	38.4 bcd
FVT-4	0.5 a	0.6 b	37.3 bcd
S2-4	0.0 b	0.7 b	32.7 cd
S2-3	0.0 b	0.0 b	32.2 d
SB-1	0.0 b	1.5 ab	11.7 e
Mean			39.3

[x]Virulence is expressed as a percent of the inoculated seedlings surviving infection.
[y]SS, RS, and RR indicate the presence or absence of the An_1 or An_2 gene for resistance to race 1 or 2, respectively.
[z]Analysis was performed on the arc sine square root transformation of the percent seedling survival means. Mean percent survival rates followed by the same letter within races and columns were not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 2. Evaluation of *Colletotrichum trifolii* race 1 isolates for ability to induce protection in alfalfa susceptible to race 2

Race 1 isolate	Resistance (percent survival)[w]		
	Race 1 on Saranac (SS)[x]	Race 1 on Arc (RS)	Race 1 then race 2 on Arc (RS)[y]
Vertbeck	0.0 d[z]	70.4 bc	55.2 a
TpRel	0.0 d	66.9 cd	50.7 ab
WL315	0.9 d	73.0 bc	49.1 ab
23R1	0.0 d	58.2 d	45.6 ab
R1 mix	0.0 d	75.7 bc	44.3 ab
3-5	1.4 d	74.7 bc	42.9 ab
271	1.8 d	73.9 bc	39.5 abc
5-3	0.7 d	70.2 bc	38.8 abc
2sp2	1.8 d	75.4 bc	38.2 abc
HN82	0.0 d	70.3 bc	34.5 bc
MDMK	5.1 bc	74.5 bc	33.6 bc
Rel	7.1 b	75.2 bc	22.3 cd
27	2.7 cd	79.6 b	15.3 de
Mean	1.7	72.1	39.2
Uninoculated	94.3 a	98.0 a	...
Race 2 only	...	...	3.4 e

[w]Resistance is expressed as a percent of the inoculated seedlings surviving infection.
[x]SS and RS indicate the presence or absence of the An_1 or An_2 gene for resistance to race 1 or race 2, respectively.
[y]Seedlings were inoculated with a mixture of race 2 isolates 24 hr after induction with race 1.
[z]Analysis was performed on the arc sine square root transformation of the percent means. Mean percent survival rates followed by the same letter within columns were not significantly different ($P = 0.05$) according to Duncan's multiple range test.

removed, inoculated with race 2 spore concentrations of 0.0, 0.25, 0.50, 1.0, 2.0, or 4.0 × 10^6 spores/ml, and returned to the mist chamber. After an additional 24 hr, pots were placed in the growth room and evaluated for disease after 5 days. Pots in the chambers were arranged in a split-plot design with four replications randomized within replication. Race 1 concentrations were the whole-plot treatments, and race 2 concentrations were the sub-plot treatments. Results from two experiments were similar, and the data presented are from a combined analysis with 0% survival values for the race 2 only treatment omitted. Data were subjected to a nonlinear weighted least squares analysis to fit the exponential function $\hat{Y} = b_0[1-\text{exponent}(b_1\ R1\ \text{concentration})]$ for the regression of race 1 and race 2 concentrations and percent resistance in Arc alfalfa (Table 3). $\hat{Y}$ is the predicted percentage of seedling survival. Confidence intervals were determined for the asymptotic percentage resistance level (b_0) and fit of the data to the exponential function (b_1) (29).

The analysis indicated a significant quantitative effect of inducer (race 1) inoculum concentration on percent survival of race 2-challenged Arc seedlings (Fig. 2 and Table 3). The higher the inducing inoculum concentration, the greater the number of seedlings surviving challenge inoculation by race 2. Resistance induced by infection of seedlings with race 1 was only slightly diminished by the increasing concentrations of challenge inoculum of race 2 up to the maximum of 4 × 10^6 spores/ml used in this experiment. This observation is supported by the overlapping confidence intervals for the asymptotic percent resistance levels among race 2 spore concentrations (Table 3). The rates of change for resistance curves for race 2 concentrations also are not significantly different (Table 3).

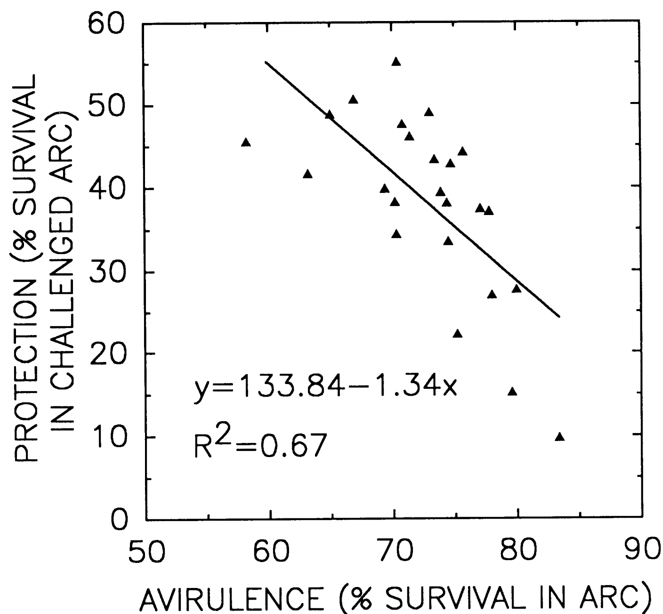

Fig. 1. Relationship between virulence of race 1 isolates of *Colletotrichum trifolii* to alfalfa cultivar Arc and the relative ability of these isolates to induce protection in Arc against challenge inoculation with race 2. Each point represents one isolate, and data from two experiments are plotted.

When protection was induced by race 1, subsequent challenge inoculation with the five concentrations of inoculum of race 2 resulted in a range of 53.3 to 64.5% survival (Table 3). These survival percentages approach the survival rates found with the expression of genetic resistance in unchallenged Arc inoculated with race 1 (71.2% survival at 4 × 10^6 spores/ml). Unprotected seedlings inoculated with race 2 alone did not survive (data not shown). These results show that, once resistance is induced, it is quite stable to challenge from high disease pressure by a highly virulent race.

Induction of anthracnose resistance by other *Colletotrichum* species. Twelve isolates and species of *Colletotrichum*, including a *C. trifolii* race 1 mixture, were used in experiments to determine whether they could protect Arc alfalfa from anthracnose caused by race 2 of *C. trifolii* (Table 4). An analysis of variance indicated no significant errors in the experiment, and data from two experiments were combined for analysis. In vitro spore germination of all isolates on potato-dextrose agar was greater than 90%. Of the isolates and species tested, only *C. trifolii* was highly pathogenic to Saranac (Table 4). One isolate of *C. gloeosporioides* (57RR) was weakly pathogenic to Saranac, permitting 76% of inoculated seedlings to survive. This isolate was highly pathogenic to Arc, killing 50% of inoculated seedlings, but did not induce protection in Arc from race 2.

Significant race 2 anthracnose resistance ($P = 0.05$) was induced in Arc by three isolates (Table 4). Race 1 of *C. trifolii* (isolate mixture) induced 36.7% resistance. Two species considered nonpathogens of alfalfa, isolate C129 of *C. gloeosporioides* and isolate 1384 of *C. malvarum*, also induced significant anthracnose resistance in Arc, permitting 19.3 and 21.0% seedling survival, respectively. Only 0.2% of unprotected race 2-inoculated seedlings survived.

Spore germination and hyphal development of all isolates were assessed on leaves taken from Arc seedlings 24 and 72 hr after inoculation. Germination and appressorium formation in all isolates and species was greater than 80% after 24 hr. After 72 hr, three isolates exhibited hyphal development from germinated spores with appressoria: isolate 57RR (20%), race 2 (23%), and race 1 (6%). Hyphal development was not observed from other isolates, including 1384 and C129. Hyphal development and penetration was not assessed beyond 72 hr.

Spore germination and hyphal development also were assessed in leaves taken from Arc seedlings that had been challenged with race 2 24 hr after inoculation with the inducing species. Penetrating hyphae were observed in leaves 72 hr after inoculation with inducing isolates 1384 (5%) and C129 (29%). However, a distinction could not be made between the hyphae of the inducing isolate and the hyphae of race 2. It is likely that the hyphae were that of race 2 because no hyphal development was observed in

TABLE 3. Nonlinear weighted least squares fit of the exponential function percent resistance = b [1 − exp ($b_1 R1$ conc.)] for the regression of race 1 and 2 spore concentrations of *Colletotrichum trifolii* and percent resistance in Arc alfalfa

Race 2 spore concentration	b_0[a] (95% confidence interval)			b_1[b] (95% confidence interval)			
(spores/ml × 10^6)	Estimate	Lower	Upper	Estimate	Lower	Upper	r^2[c]
0.25	64.5	59.3	69.8	−1.5	−1.2	−1.9	0.87
0.50	59.8	55.2	64.3	−1.5	−1.2	−1.8	0.89
1.00	54.7	49.9	59.5	−1.7	−1.3	−2.2	0.83
2.00	57.4	52.7	62.0	−1.8	−1.4	−2.3	0.86
4.00	53.3	48.3	58.4	−1.7	−1.2	−2.2	0.83
Combined data	57.9	55.7	60.1	−1.6	−1.5	−1.8	0.85

[a] b_0 is the asymptotic percent resistance level.
[b] b_1 ($-b_0b_1$ exp [b_1 $R1$ conc.]) defines the rate of change in percent resistance as the race 1 spore concentration is changed. Race 1 concentrations were 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 × 10^6 spores/ml.
[c] $r^2 = 1 -$ RSS/CTSS. RSS is the residual sum of squares, and CTSS is the corrected total sum of squares.

leaves 72 hr after inoculation with the inducing isolate alone. Given the genetically heterogeneous nature of Arc, it also was not possible to determine whether the seedlings selected for examination contained genes for resistance to anthracnose.

DISCUSSION

Induced resistance is expressed in alfalfa as an increase in the number of seedlings surviving inoculation with a compatible pathogen (race 2), which kills virtually all unprotected seedlings. This criterion of percent survival commonly is used in describing genetic resistance to anthracnose in alfalfa germ plasm (26). Our results demonstrate that, by using standardized resistance evaluation procedures, significant protection against race 2 of *C. trifolii* was easily induced in seedlings and the degree of protection was correlated with increasing inducer inoculum concentrations (Fig. 1). In addition, protection was not broken down by high levels of disease pressure, even when seedlings were challenged by high race 2 inoculum levels (4×10^6 spores/ml). Dean and Kuć (5) reported similar results for cucumber, where systemic protection induced by *C. lagenarium* was not overcome by high levels of challenge inoculum.

Welty and Rawlings (35) investigated the effect of inoculum concentration of *C. trifolii* on disease severity in alfalfa, finding that increasing race 1 or race 2 spore concentrations from 1×10^3 to 1×10^6 spores/ml resulted in increasing disease severity on cultivars resistant or susceptible to anthracnose. Our results suggest that, in addition to the expected relationship between inoculum density and disease severity, the degree of protection is a function of concentration of inducing inoculum. Of importance is the finding that, depending on inoculum source and concentration, the levels of resistance that were induced were very high, nearly as great but never exceeding resistance levels expected from genetic resistance alone (Fig. 2, Table 2). In the induced protection experiment (Fig. 2), low inoculum concentrations of race 1 only (0.125×10^6 and 0.25×10^6 spores/ml) resulted in an unexpectedly lower percent survival of seedlings than those inoculated with higher inoculum concentrations of race 1. This was observed in both runs of the experiment. This phenomenon may reflect a lack of sufficient inoculum to activate the defense response, or possibly a lag time before fungitoxic levels of phytoalexins accumulate over sufficient area to inhibit further infection.

Significant variation in isolate virulence was found in this study (Table 1). This variation in the ability of isolates to kill alfalfa seedlings would have a definite effect on breeding programs for disease resistance. Consequently isolates used repeatedly in breeding programs should be monitored for virulence. When monitoring is not feasible, isolate mixtures should be used. In histological studies on Stemphylium leaf spot of alfalfa, Cowling and Gilcrest (4) found that differences in relative virulence of isolates correlated with the frequency of stomatal penetration. In critical experiments, they demonstrated independent expression of relative virulence and relative resistance. In earlier work we found no differences between races in appressorium formation and penetration frequency (3); however, we did not examine isolate differences. In studies with mutants of *Botrytis fabae* Sardiña differing in virulence to broad bean, Hutson and Mansfield (17) determined that the major determinant of virulence in this species is the relative ability of isolates to kill epidermal cells soon after penetration, thereby suppressing phytoalexin accumulation. They noted, however, that a degree of tolerance to the phytoalexin wyerone acid also may be an essential requirement for pathogenicity.

Relative isolate virulence in *Nectria haematococca* Berk. & Br. has been associated with phytoalexin tolerance and the ability of these isolates to demethylate the phytoalexin pisatin (33,34). In this system, spore progeny segregating from sexual crossings, isolates with diverse origins, and transformants selected for their ability to demethylate pisatin were evaluated for tolerance to pisatin, which was associated with their with relative virulence to pea (*Pisum sativum* L.) (33,34,37). Spore progeny from matings are not currently available for *C. trifolii* because the perfect state of this pathogen has not been induced in culture. However, a range in virulence among race 2 field isolates and the clear distinction between races on specific host genotypes (Table 1) may provide enough variation to examine possible correlative relationships with virulence.

The association between relative virulence of race 1 isolates to Arc and their relative ability to induce resistance to race 2 (Fig. 2) suggests that fungal components determining virulence and induction may function similarly by activating the An_1 gene for resistance to race 1. This explanation is reasonable because it was determined earlier that the presence of a gene for anthracnose resistance is a requirement for the expression of induced protection (25), and protection induced in this study did not exceed natural genetic resistance typical of Arc. However, further correlative relationships between virulence and both types of resistance should be investigated at the histological and biochemical levels to test the hypothesis that fungal attributes or components determining virulence or aggressiveness are similar to those effective in inducing resistance.

Induced resistance in plants is a well-known phenomenon, but the mechanisms involved are only partly known and may function differently from genetic resistance. We demonstrated earlier (3) that resistance to anthracnose was not associated with the failure of conidia to germinate, form appressoria, or penetrate resistant tissues. The expression of resistance occurred near and at the time of epidermal penetration. Preliminary evidence suggests that natural and possibly induced resistance is associated with the accumulation of lethal concentrations of the phytoalexins in specific resistant genotypes and that race 1 and race 2 of *C. trifolii* may be differentially tolerant to the phytoalexin medicarpin (21, and O'Neill and Baker, *unpublished*). The observation that the degree of protection is related to the concentration of race 1 inoculum concentration used for induction (Fig. 2) would support the hypothesis of quantitative accumulation of phytoalexins from increasing numbers of infection sites. However, the mechanism of induced protection may be controlled or may function differently in different tissues from the mechanism for natural resistance. In French bean, for example, tissues protected by an incompatible race of *C. lindemuthianum* were protected from the compatible race at a later stage of infection development than in naturally

TABLE 4. Virulence of species of *Colletotrichum* to alfalfa cultivars Arc and Saranac, and capacity to induce protection in Arc from challenge inoculation by *C. trifolii* race 2

Colletrotrichum species and isolate designation	Virulence[a] (% survival)		Protection[b] (% survival)
	Saranac	Arc	Arc challenged with race 2
C. coccodes (C4)	91.4 a[c]	93.3 a	3.4 c
C. coccodes (C9)	90.3 a	90.1 a	1.2 c
C. atramentarium (C111)	89.5 a	89.6 a	1.2 c
C. atramentarium (C113)	96.0 a	95.3 a	1.7 c
C. dematium (C59)	91.5 a	89.0 a	2.6 c
C. destructivum (C108)	92.8 a	89.2 a	2.1 c
C. gloeosporioides (C129)	96.7 a	94.5 a	19.3 b
C. malvarum (1384)	92.7 a	94.7 a	21.0 b
C. gloeosporioides (47RR)	89.0 a	85.8 a	2.0 c
C. gloeosporioides (57RR)	76.0 b	50.3 c	3.6 c
C. spp. (3)	87.7 a	88.4 a	6.4 c
C. trifolii (race 1 mix)[d]	0.7 c	69.6 b	36.7 a
C. trifolii (race 2 mix)[e]	...	...	0.2 c
Noninoculated	95.6 a	91.1 a	...

[a]Virulence is expressed as a percent of the inoculated seedlings surviving infection.
[b]Protection is expressed as a percent of the inoculated seedlings surviving challenge inoculation by race 2 of *C. trifolii*.
[c]Analysis was performed on the arc sine square root transformation of the percent means. Mean percent survival rates followed by the same letter within columns were not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[d]A mixture of eight race 1 isolates.
[e]A mixture of 12 race 2 isolates.

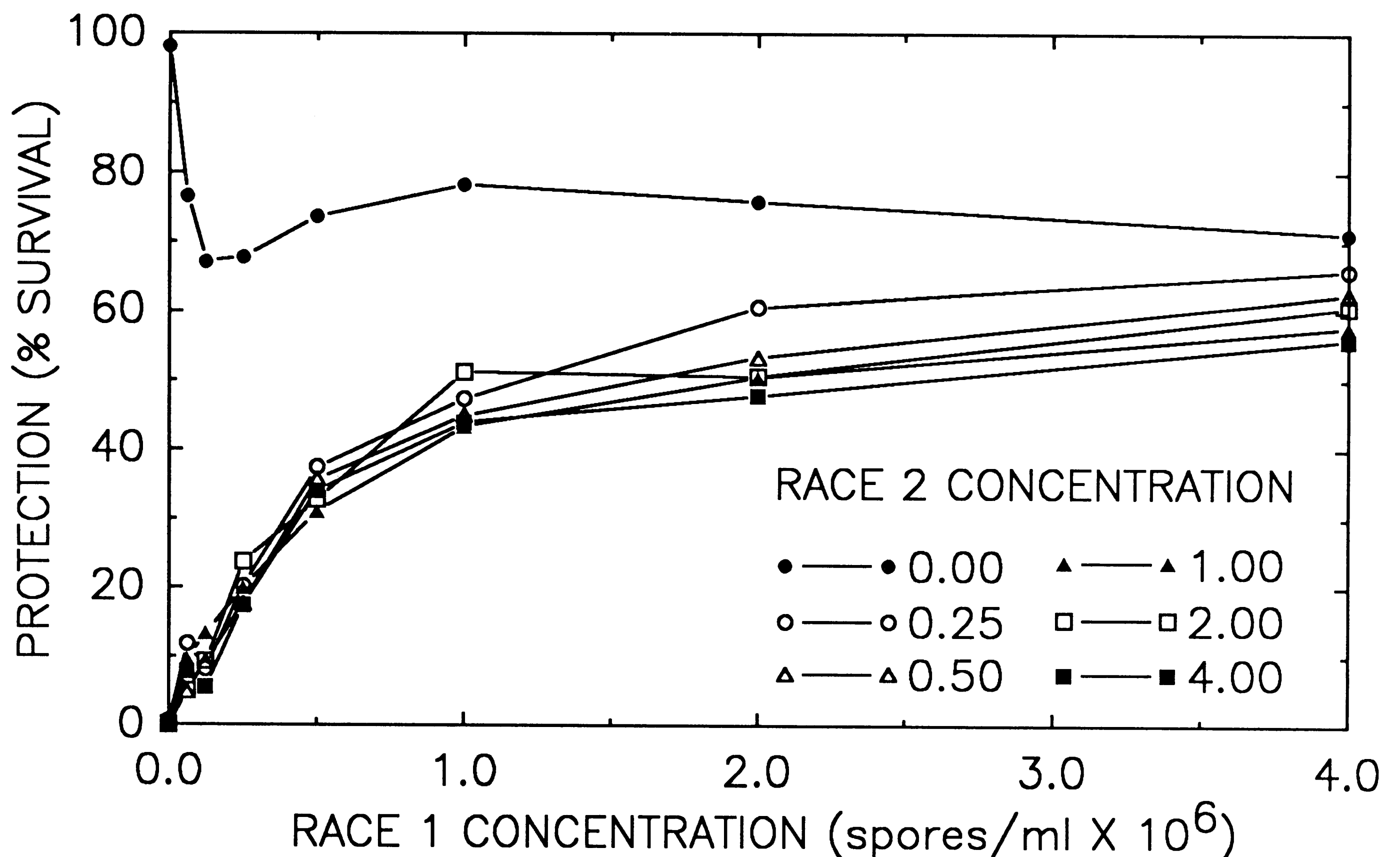

Fig. 2. Effect of *Colletotrichum trifolii* race 1 inoculum concentration on resistance induced in alfalfa cultivar Arc seedlings to anthracnose caused by race 2. The effect of challenge inoculum concentrations of race 2 (0.00 to 4.00 $\times$ 10^6 spores/ml) was assessed by inoculating seedlings with race 2 24 hr after inoculation with race 1. Induced protection is expressed as a percent of the seedlings surviving the induction and challenge inoculations. No seedlings survived inoculation with race 2 alone. Percent survival of seedlings inoculated with race 1 alone is typical for the Arc cultivar.

resistant tissues (12,31).

The enhancement of resistance may be of considerable economic value if it is exploited for field use. The finding that protection can be induced by certain nonpathogenic *Colletotrichum* species or isolates (Table 4) broadens the possibilities for developing a practical induced resistance system. If species of *Colletotrichum* could be found that were not major pathogens of other crops, they could be used, as suggested by Graham et al (16), for inducing resistance.

Comprehensive investigations of the temporal relationships among germination, fungal ingress, phytoalexin production, protection duration, and phytoalexin tolerance are necessary to identify and separate the expression of relative virulence and resistance, and to identify the mechanisms of natural and induced resistance to anthracnose of alfalfa.

LITERATURE CITED

1. Barnes, D. K., Ostazeski, S. A., Shillinger, J. A., and Hanson, C. H. 1969. Effect of anthracnose (*Colletotrichum trifolii*) infection on yield, stand, and vigor of alfalfa. Crop Sci. 9:344-346.
2. Caruso, F. L., and Kuć, J. 1977. Protection of watermelon and muskmelon against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. Phytopathology 67:1285-1289.
3. Churchill, A. C. L., Baker, C. J., O'Neill, N. R., and Elgin, J. H., Jr. 1988. Development of *Colletotrichum trifolii* races 1 and 2 on alfalfa clones resistant and susceptible to anthracnose. Can. J. Bot. 66:75-81.
4. Cowling, W. A., and Gilcrest, D. G. 1982. Expression of pathogen virulence and host resistance during infection of alfalfa with *Stemphylium botryosum*. Phytopathology 72:36-42.
5. Dean, R. A., and Kuć, J. 1986. Induced systemic protection in cucumber: Effects of inoculum density on symptom development caused by *Colletotrichum lagenarium* in previously infected and uninfected plants. Phytopathology 76:186-189.
6. Devine, T. E., and McMurtrey, J. E., III. 1975. Performance of anthracnose-resistant alfalfa strains. Crop Sci. 15:505-508.
7. Elgin, J. H., Jr., Barnes, D. K., Busbice, T. H., Buss, G. R., Clark, N. A., Cleveland, R. W., Ditterline, R. L., Evans, D. W., Fransen, S. C., Horrocks, R. D., Hunt, O. J., Kehr, W. R., Lowe, C. C., Miller, D. A., Offutt, M. S., Pickett, R. C., Sorrensen, E. L., Taliaferro, C. M., Tesar, M. B., and Van Keuren, R. W. 1981. Anthracnose resistance increases alfalfa yields. Crop Sci. 21:457-460.
8. Elgin, J. H., Jr., and O'Neill, N. R. 1988. Comparison of genes controlling race 1 anthracnose resistance in Arc and Saranac AR alfalfa. Crop Sci. 28:657-659.
9. Elgin, J. H., Jr., and Ostazeski, S. A. 1982. Evaluation of selected alfalfa cultivars and related *Medicago* species for resistance to race 1 and race 2 anthracnose. Crop Sci. 22:39-42.
10. Elgin, J. H., Jr., and Ostazeski, S. A. 1985. Inheritance of resistance to race 1 and race 2 anthracnose in Arc and Saranac AR alfalfa. Crop Sci. 25:861-865.
11. Elliston, J. E., Kuć, J., and Williams, E. B. 1971. Induced resistance to bean anthracnose at a distance from the site of inducing interaction. Phytopathology 61:1110-1112.
12. Elliston, J. E., Kuć, J., and Williams, E. B. 1976. A comparative study of the development of compatible, incompatible, and induced incompatible interactions between *Colletotrichum* spp. and *Phaseolus vulgaris*. Phytopathol. Z. 87:289-303.
13. Elliston, J. E., Kuć, J., Williams, E. B., and Rahe, J. E. 1977. Relationship of phytoalexin accumulation to local and systemic protection of bean against anthracnose. Phytopathol. Z. 88:114-130.
14. Engesser, R., and Gessler, C. 1987. The ability of a cultivar pathogenic race of *Colletotrichum lindemuthianum* to induce resistance to bean anthracnose in etiolated bean hypocotyls. Bot. Helv. 97:341-348.
15. Graham, J. H. 1979. A Compendium of Alfalfa Diseases. American Phytopathological Society, St. Paul, MN. 65 pp.
16. Graham, J. H., Devine, T. E., and Hansen, C. H. 1976. Occurrences and interaction of three species of *Colletotrichum* on alfalfa in the Mid-Atlantic United States. Phytopathology 66:538-541.
17. Hutson, R. A., and Mansfield, J. W. 1980. A genetical approach to the analysis of mechanisms of pathogenicity in *Botrytis/Vicia faba* interactions. Physiol. Plant Pathol. 17:309-317.
18. Kuć, J. 1983. Induced systemic resistance in plants to diseases caused

by fungi and bacteria. Pages 191-221 in: The Dynamic of Host-Defense. J. A. Bailey and B. J. Deverall, eds. Academic Press, Sidney.
19. Kuć, J., Schockley, G., and Kearney, K. 1975. Protection of cucumber against *Colletotrichum lagenarum* by *Colletotrichum lagenarum*. Physiol. Plant Pathol. 7:195-199.
20. Mansfield, J. W. 1982. The role of phytoalexins in disease resistance. Pages 253-288 in: Phytoalexins. J. A. Bailey and J. M. Mansfield, eds. Blackie & Son Ltd., Glasgow. 334 pp.
21. O'Neill, N. R., Baker, C. J., and Campbell, T. A. 1987. Accumulation of medicarpin in resistant and susceptible alfalfa seedlings and fungitoxicity to *Colletotrichum trifolii*. (Abstr.) Phytopathology 77:1751.
22. O'Neill, N. R., Elgin, J. H., Jr., and Baker, C. J. 1986. Effects of inoculum concentration and virulence of *Colletotrichum trifolii* race 1 and 2 isolates on induced protection in *Medicago sativa*. (Abstr.) Phytopathology 76:1143.
23. Ostazeski, S. A., and Elgin, J. H., Jr. 1980. Physiologic races of *Colletotrichum trifolii*. (Abstr.) Phytopathology 70:691.
24. Ostazeski, S. A., and Elgin, J. H., Jr. 1982. Use of hypodermic inoculations of alfalfa for identifying host reactions and races of *Colletotrichum trifolii*. Crop Sci. 22:545-546.
25. Ostazeski, S. A., and Elgin, J. H., Jr. 1984. Resistance induced by race 1 of *Colletotrichum trifolii* to race 2 in alfalfa resistant to race 1. Plant Dis. 68:285-288.
26. Ostazeski, S. A., and Elgin, J. H., Jr. 1984. Standard tests to characterize pest resistance in alfalfa cultivars. U.S. Agric. Res. Serv. Misc. Publ. 1434. 38 pp.
27. Porto, M. D. M., Grau, C. R., de Zoeten, G. A., and Gaard, G. 1988. Histopathology of *Colletotrichum trifolii* on alfalfa. Phytopathology 78:345-349.
28. Rahe, J. E., Kuć, J., Chuang, C., and Williams, E. B. 1969. Induced resistance in *Phaseolus vulgaris* to bean anthracnose. Phytopathology 59:1641-1645.
29. SAS Institute. 1985. SAS User's Guide: Statistics. 5th ed. SAS Institute, Inc., Cary, NC. 956 pp.
30. Sequeira, L. 1983. Mechanisms of induced resistance in plants. Annu. Rev. Microbiol. 37:51-79.
31. Skipp, R. A., and Deverall, B. J. 1973. Studies on cross-protection in the anthracnose disease of bean. Physiol. Plant Pathol. 3:299-314.
32. Svoboda, W. E., and Paxton, J. D. 1972. Phytoalexin production in locally cross-protected Harosoy and Harosoy-63 soybeans. Phytopathology 62:1457-1460.
33. Tegtmeier, K. J., and Van Etten, H. D. 1982. The role of pisatin tolerance and degradation in the virulence of *Nectria haematococca*. A genetic analysis. Phytopathology 72:607-612.
34. Van Etten, H. D., Matthews, P. S., Tegtmeier, K. J., Dietart, M. F., and Stein, J. L. 1980. The association of pisatin tolerance and demethylation with virulence on pea in *Nectria haematococca*. Physiol. Plant Pathol. 16:257-268.
35. Welty, R. E., and Mueller, J. P. 1979. Occurrence of a highly virulent isolate of *Colletotrichum trifolii* on alfalfa in North Carolina. Plant Dis. Rep. 63:666-670.
36. Welty, R. E., and Rawlings, J. O. 1985. Effects of inoculum concentration and temperature on anthracnose severity in alfalfa. Phytopathology 75:593-598.
37. Yoder, O. C., Weltring, K., Turgeon, B. G., Garber, R. C., and Van Etten, H. D. 1986. Technology for molecular cloning of fungal virulence genes. Pages 371-384 in: Biology and Molecular Biology of Plant Pathogen Interactions. J. A. Bailey, ed. Springer-Verlag, Heidelberg.