Resistance Induced by Race 1 of *Colletotrichum trifolii* to Race 2 in Alfalfa Resistant to Race 1

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ABSTRACT

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Alfalfa plants resistant to race 1 but susceptible to race 2 of *Colletotrichum trifolii* could be protected from race 2 when needle-inoculated with mixtures of races 1 and 2. Dilution was not a factor because race 2 alone at 1×10^6 conidia per milliliter was pathogenic but the race 2 component in the mixture at 4×10^6 conidia per milliliter was relatively, or usually, completely nonpathogenic. In spray-inoculation experiments, most cultivar Arc seedlings were protected from race 2 by prior inoculation with race 1. The survival rate of Arc seedlings inoculated with a mixture of races 1 and 2 was much higher than that of seedlings inoculated with race 2 alone. Analysis of plants capable of being protected from race 2 in mixtures, or by protective sprays with race 1, demonstrated race 1 resistance was a requirement for the race 2 protection phenomenon to be operative.

During the late 1960s, techniques were developed for isolating resistance in alfalfa (*Medicago sativa* L.) to anthracnose caused by *Colletotrichum trifolii* Bain (9). Soon highly resistant cultivars such as Arc and Saranac AR were released. More recently, other cultivars with varying levels of anthracnose resistance have become common in the seed trade (3).

During the summer of 1978, a second race of *C. trifolii* was found in Maryland and North Carolina that rendered some cultivars, previously anthracnose resistant (10,16), ultrasusceptible. In our early attempts to isolate resistance per se to both races of the pathogen (mainly from Arc or Arc-related populations), inoculum of both races was combined. Consistently higher numbers of survivors resulted from such inoculations than would have been expected from the same populations inoculated with race 2 alone. Similarly, we noted a lack of symptom development, or at least a significant decrease in lesion size, when certain clones known to be susceptible to race 2 were inoculated with conidial mixtures of races 1 and 2 via hypodermic syringes (11).

The experiments reported in this paper showed that certain alfalfa clones, and most seedlings from the cultivar Arc, lacking specific resistance to race 2 of *C. trifolii* can be protected against race 2 by prior inoculation with race 1 of the same organism. Preliminary reports of these studies have been published (11,12).

MATERIALS AND METHODS

Inoculum was produced on plates of half-strength oatmeal agar (36 g Difco oatmeal agar, 1 L distilled water, and 7 g Difco agar). Inoculum was harvested in 7–10 days by loosening conidia with a camel's hair brush in Tween 20 solution (two drops of Tween 20 per liter of water). Conidial concentrations for injection inoculations (13) ranged from 1 to 9×10^6 conidia per milliliter. Infections from injection inoculations were scored after 10–14 days on a basis of 1–3, where 1 = no symptoms or only a slight discoloration; 2 = an intermediate reaction where lesions were small, nonsporulating, and often highly pigmented (purple); and 3 = a large, often stem-girdling, sporulating lesion.

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Inoculum for spray inoculations was standardized at 2×10^6 conidia per milliliter and applied at the rate of 50 ml/flat (40 × 55 cm) or its equivalent. In these experiments, seedling survivors were counted and numbers converted to percentage of the original stand. Plant material to be inoculated was from three sources:

1. *Surviving Arc plants.* Forty surviving plants were retained from a potential 17,000+ Arc population grown in 24 flats (720 scarified seeds per flat). This planting had been spray-inoculated twice with a mixture of races 1 and 2. Spray inoculations were followed later by repeated needle inoculations with race 1 and/or a mixture of races 1 and 2. Following a careful roguing of susceptible plants, survivors were cut back and transplanted singly to 15-cm clay pots, assigned plant numbers, and needle-inoculated with race 2 (2×10^6 conidia per milliliter) or a mixture of races 1 and 2 (1×10^6 conidia of each race per milliliter).

2. *Standard clones.* The use of our standard clones, originating from Arc or Saranac AR sources, has been described elsewhere (13,14). Phenotypic reactions to races 1 and 2 of these clones have been verified repeatedly. To rule out a possible dilution effect in the mixed inoculum administered via injection, dilutions of race 1, race 2, and the mixture of races 1 and 2 were administered to our standard anthracnose clones (14) in addition to some of the aforementioned select Arc plants. All experiments involving injection inoculations were conducted in a greenhouse at 26 ± 7 C.

3. *Arc seed.* Two seedling experiments were conducted to determine whether induced resistance would be limited to needle-inoculated mature plants. Arc seeds were sown 50 seeds per 10-cm plastic pot in our standard greenhouse potting mixture. Stand counts were made 7–10 days after seeding and again 10–14 days after inoculation. In the first experiment, 36 pots of 2-wk-old Arc seedlings were given a protective spray inoculation with race 1 (seedlings hereafter referred to as "protected"). Six hours later, 12 pots each of protected and unprotected seedlings were challenged with a spray inoculation of race 2. Similarly, 12 and 24 hr after the protective inoculation with race 1, 12 pots each of the protected and unprotected seedlings were challenged with race 2.

A second spray-inoculation experiment was conducted in which the race 2 challenge was made at 0, 1, 2, 4, 8, 16, and up to 256 hr after the race 1 protective inoculation. In this experiment, the final stand count was made 14 days after the final inoculation in the experimental series (the 256-hr race 2 challenge).

The first seedling spray-inoculation experiment was conducted in a growth room (24 ± 3 C). The second seedling spray-inoculation experiment was grown in the same growth room until all inoculations were completed, then moved to the greenhouse.

Protected survivors from the second seedling spray-inoculation experiment were transplanted singly to 10-cm clay pots. After plants had attained sufficient growth, each was needle-inoculated with race 1, scored for reaction, cut back, and fertilized. Again, after most of the plants had attained sufficient regrowth, they were needle-inoculated with race 2 and scored for reaction.

Table 1. Reactions of select cultivar Arc alfalfa plants needle-inoculated with race 2 or a mixture of races 1 and 2 of *Colletotrichum trifolii*

Plant	Race 2[a]	Mixture
1	2.7[b]	3.0
2	1.0	1.0
3	2.7	1.7
4	1.7	1.0
5	2.3	1.0
6	2.7	1.0
7	3.0	1.0
8	1.3	1.0
9	2.0	1.0
10	2.3	1.0
11	2.7	1.0
12	1.0	1.0
13	3.0	1.0
14	2.0	1.0
15	1.0	1.0
16	1.7	1.0
17	2.3	1.0
18	3.0	1.0
19	2.3	1.0
20	1.0	1.0
21	2.3	1.0
22	1.0	1.0
23	2.3	1.0
24	1.0	1.0
25×75[c]	3.0	3.0

[a] Conidial concentration of race 2 is 2×10^6/ml. Concentration of the mixture is 1×10^6 of each race per milliliter.
[b] Average score for three sites 10–14 days after inoculation, where 1 = no symptoms; 2 = small to medium area of discoloration, no sporulation; and 3 = large lesion, many acervuli.
[c] Clone 25×75 is known to be susceptible to both race 1 and race 2.

RESULTS AND DISCUSSION

From the 40 surviving Arc plants, 24 were selected for further study. Table 1 lists reactions for each plant × inoculum treatment. One plant (no. 1) proved to be a susceptible escape (inadvertently carried through all previous inoculations) and was susceptible to both race 2 and the mixed inoculum. Similarly, a known anthracnose-susceptible control, clone 25×75, was also susceptible to both inocula. Nine plants were classed as resistant to both inocula. Most of the assayed plants were susceptible to race 2 inoculum but resistant to the mixture.

It is clear that certain Arc plants susceptible to race 2 (Table 2, nos. 3, 6, 11, 13, and 21) and clonal propagules of some of the standard clones (Table 3, clones 16 RS and 54 RS) included in these experiments are protected from the race 2 component of a mixed inoculum. Resistance shown by these plants to the race 2 component in mixtures of inoculum cannot be attributed to dilution because race 2 inoculum at its lowest concentrations was pathogenic, whereas the race 2 component in the mixed inoculum was nonpathogenic at a concentration four times higher than the lowest concentration of race 2 inoculum. Susceptible clones 25×75 (Table 2) and 4 SS and 31 SS (Table 3) were susceptible to all inocula at all concentrations.

Data from seedling experiment 1 are presented in Table 4. In this experiment, most of the Arc seedlings, though injured (Fig. 1), were dramatically protected from race 2 by a prior protective inoculation with race 1. The level of protection from the race 2 challenge apparently would have peaked sometime beyond 24 hr after the protective inoculation.

In the second seedling experiment, the protection peaked at 8 hr (Fig. 2), but its effect was apparent even 128 hr after the race 1 protective spray inoculation. Of equal biological significance is the fact that at time zero, the mean survival rate for seedlings inoculated with mixed inoculum was higher than for seedlings

Table 2. Reactions of select cultivar Arc alfalfa plants needle-inoculated with race 1, race 2, or a mixture of races 1 and 2 of *Colletotrichum trifolii* at three conidial concentrations

	Conidial concentration (no./ml)								
	Race 1			Race 2			Mixture[a]		
Plant	1×10^6	2×10^6	4×10^6	1×10^6	2×10^6	4×10^6	1×10^6	2×10^6	4×10^6
3	1.0[b]	1.0	1.0	2.3	2.3	2.3	1.0	1.0	1.0
6	1.0	1.0	1.0	3.0	2.7	2.7	1.0	1.3	1.3
11	1.0	1.0	1.0	3.0	2.7	3.0	1.0	1.0	1.0
13	1.0	1.0	1.0	3.0	1.7	2.3	1.0	1.0	1.0
20	1.0	1.0	1.0	1.3	1.7	1.3	1.0	1.0	1.0
21	1.0	1.0	1.0	2.7	3.0	2.7	1.0	1.0	1.0
24	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
25×75[c]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0

[a] Conidial concentration of each mixture is that of each race per milliliter.
[b] Average score for three sites 10–14 days after inoculation, where 1 = no symptoms; 2 = small to medium area of discoloration, no sporulation; and 3 = large lesion, many acervuli.
[c] Clone 25×75 is known to be susceptible to both race 1 and race 2.

inoculated with race 2 alone. These data explain, at least in part, some of the vagaries we experienced early in our work with race 2, where use of combined inoculum of races 1 and 2 led to unusually high survival rates of some alfalfa populations.

From the second seedling spray-inoculation experiment, there were 162 plants available that had survived a protective inoculation of race 1 followed by a challenge inoculation with race 2. Of these, 161 (99.3%) proved to be resistant to race 1 when they were inoculated via the needle injection method. After the race 1 evaluation, 156 plants remained for a race 2 inoculation. Of these, only five proved resistant to race 2 (3.2%). Thus, it appears that the requirement for induced resistance to race 2 to be operative in the alfalfa used in these experiments is resistance to race 1 (Table 2, and clones 16 RS and 54 RS in Table 3).

The phenomenon of protection (induced resistance) in crop plants against certain pathogens is well known. This protection includes resistance induced by the same pathogen (1,7), by different races of the same pathogen (2), by different pathogens (5), and by other crop pathogens nonpathogenic to the protected host (4). To our knowledge, such induced resistance has not been described before in forage legumes. However, a phytoalexin has been associated with resistance to Phytophthora root rot of alfalfa (15) and has been shown to be produced in diffusates on detached leaves inoculated with alfalfa pathogens and nonpathogens (6).

A chemical (that is, phytoalexin) explanation for induced resistance seems plausible in these experiments for several reasons. First, in our experiments, race 2 inoculum was rendered innocuous when needle-inoculated into stems of plants known to be resistant to race 1 but was susceptible to race 2 *if* in a mixture with an "eliciting" race 1. Second, race 2 was rendered less pathogenic on Arc seedlings when such seedlings were challenged (in spray inoculations) with a mixture of races 1 and 2 conidia. Arc populations ordinarily have 65–75% of the members highly resistant to race 1 but often have less than 1% resistant to race 2 (3). Third, the protection afforded Arc seedlings was effective up to 128 hr prior to a race 2 challenge and dissipated thereafter. Fourth, the Arc seedlings analyzed after surviving a protective inoculation with race 1 and a challenge inoculation with race 2 were almost 100% resistant to race 1. Again, this indicates the prerequisite that resistance to race 1 must be inherent for the system to be operative, at least in Arc alfalfa.

Kuć et al (8) alluded to the possible role of such protection phenomena under field conditions. In the Middle Atlantic states, anthracnose is seldom a problem on susceptible alfalfas until mid- to late July, despite the fact that primary inoculum is assumed to be present. Cool temperature before this time should be ruled out as a reason for a lack of disease incidence, because in our experience, efficient screening for anthracnose resistance can be accomplished in the inoculation chamber at temperatures of 20 C and lower. Spring blackstem caused by *Phoma medicaginis* Malbr. & Roum.

Table 3. Reactions of standard alfalfa clones needle-inoculated with race 1, race 2, or a mixture of races 1 and 2 of *Colletotrichum trifolii* at three conidial concentrations

	Conidial concentration (no./ml)								
	Race 1			Race 2			Mixture[a]		
Plant	1×10^6	2×10^6	4×10^6	1×10^6	2×10^6	4×10^6	1×10^6	2×10^6	4×10^6
4 SS	2.5[b]	3.0	3.0	3.0	3.0	2.5	2.5	2.5	2.5
16 RS	1.0	1.0	1.0	3.0	3.0	3.0	2.0	2.0	1.0
31 SS	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
54 RS	1.0	1.0	1.0	3.0	3.0	2.5	1.0	1.0	1.0
1 RR	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] Conidial concentration of each mixture is that of each race per milliliter.
[b] Average score for three sites 10–14 days after inoculation, where 1 = no symptoms; 2 = small to medium area of discoloration, no sporulation; and 3 = large lesion, many acervuli.

Table 4. Percent survival of cultivar Arc alfalfa seedlings as influenced by a protective spray inoculation with race 1 before challenge spray inoculations with race 2 of *Colletotrichum trifolii*

	Inoculation treatments			
Time of treatment	Race 1, then race 2	Race 2 alone	Race 1 alone	Uninoculated control
At time zero	...	...	47.4	74.5
After 6 hr	56.2	1.5	...	...
After 12 hr	58.1	2.9	...	...
After 24 hr	69.9	6.5	...	...

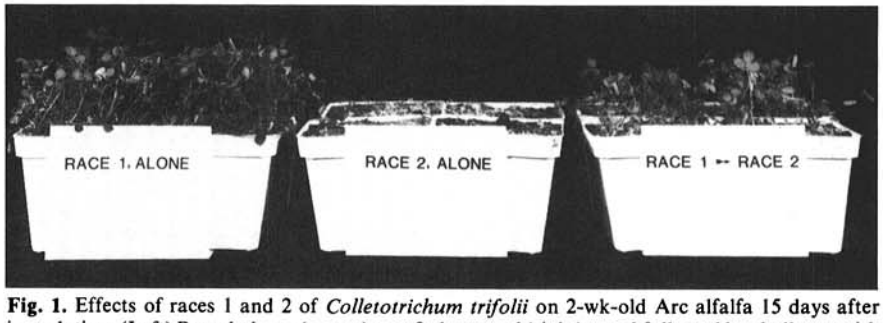

Fig. 1. Effects of races 1 and 2 of *Colletotrichum trifolii* on 2-wk-old Arc alfalfa 15 days after inoculation. (Left) Race 1 alone, (center) race 2 alone, and (right) race 1 followed by challenge with race 2 after 18 hr.

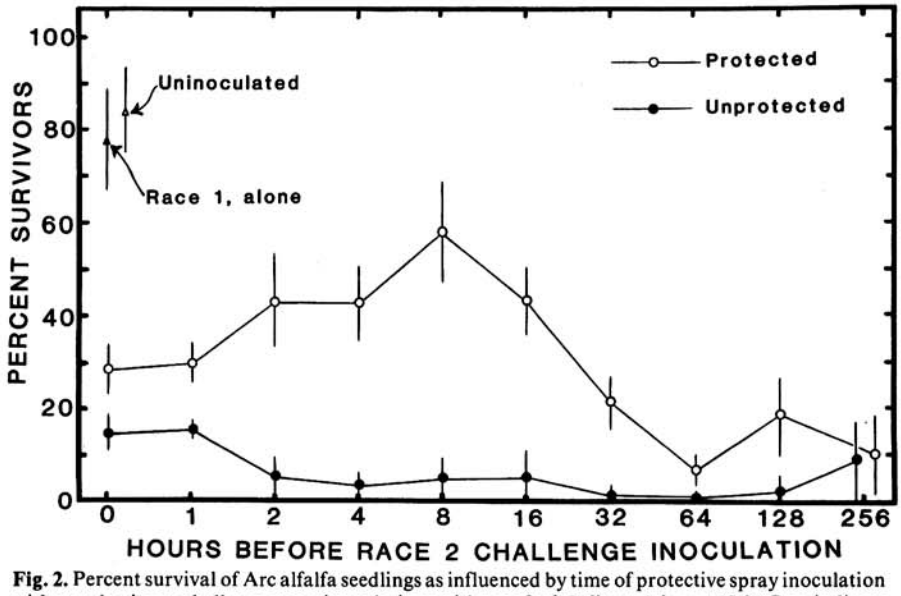

Fig. 2. Percent survival of Arc alfalfa seedlings as influenced by time of protective spray inoculation with race 1 prior to challenge spray inoculations with race 2 of *Colletotrichum trifolii*. Bars indicate standard errors.

var. *medicaginis* Boerema occurs early in the season. It is conceivable that the blackstem organism, as well as others present in the field during the spring, could elicit some resistance to anthracnose through a protection phenomenon similar to the one we have described, ie, induced resistance. It is also conceivable that this induced resistance would only be negated later by some factor(s) in the environment.

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