Evaluation of Infection of Target and Nontarget Hosts by Isolates of the Potential Biocontrol Agent *Puccinia jaceae* that Infect *Centaurea* spp.

N. Shishkoff and W. Bruckart

Research affiliate and research plant pathologist, U.S. Department of Agriculture, Agricultural Research Service, Foreign Disease-Weed Science Research, Bldg. 1301, Ft. Detrick, Frederick, MD 21702.

Present address of first author: Dynamac Corp., 2275 Research Blvd., Rockville, MD 20850-3268.

We are grateful to Dave Koogle at USDA, Frederick, MD, for laboratory and technical assistance, Craig Cavin and Rick Bennett at USDA, Frederick, MD, for generously sharing their rust isolates, David Supkoff at the California Department of Food and Agriculture (CDF&A) and Cindy Roche at Washington State University for procurement of seeds, and Will Potts at USDA, Beltsville, MD, for advice on the statistical treatment of data. Financial support was provided by CDF&A Agreement No. 58-3615-0-21.

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Accepted for publication 3 May 1993.

ABSTRACT

Shishkoff, N., and Bruckart, W. 1993. Evaluation of infection of target and nontarget hosts by isolates of the potential biocontrol agent *Puccinia jaceae* that infect *Centaurea* spp. Phytopathology 83:894-898.

In order to evaluate damage caused by the rust *Puccinia jaceae* to target and nontarget plants, number of pustules per leaf, dry root weight, and rate of leaf senescence were assessed. Each of five rust isolates was inoculated onto the host from which it was collected (yellow starthistle, purple starthistle, or diffuse knapweed) and also onto a nontarget host (cornflower) that is susceptible to all five isolates. Skeleton weed inoculated with *P. chondrillina* was included in the study as a comparable successful biocontrol system. On target hosts, there was no significant correlation between number of pustules and root biomass, which suggests that disease severity assessments based on pustule counts alone are a weak measure of biocontrol potential. When whole plants were inoculated, isolate YST71 caused a significant reduction in the root biomass of yellow starthistle, but other isolates of *P. jaceae* had little effect on their target hosts. The root biomass of yellow starthistle and that of diffuse knapweed were reduced significantly with inoculation of up to eight leaves, whereas no significant reduction of root biomass was seen in purple starthistle. When *P. jaceae* was inoculated onto cornflower, infections caused by the five isolates did not differ in severity when measured as pustules per leaf and caused no reduction of root biomass. The differences in infection-induced leaf senescence among target hosts and cornflower appeared host-related, which suggests that characteristics of plants, such as the number of leaves per rosette and a healthy leaf lifespan, may influence their responses to infection. The response of yellow starthistle to *P. jaceae* in greenhouse tests was comparable to that of skeleton weed to *P. chondrillina.*

Yellow starthistle (*Centaurea solstitialis* L.) is an introduced winter annual with allelopathic properties that displaces valuable forage plants in the western United States (6). Purple starthistle (*C. calcitrapa* L.) is a biennial from the Mediterranean area that was introduced into California early in this century (1). Diffuse knapweed (*C. diffusa* Lam.) is a biennial to triennial herb from Eurasia (16). All are weeds that are targeted for biocontrol with rust fungi. Rusts are considered to be good candidates for use as biological control agents because of their host specificity and airborne spores. Isolates of *Puccinia chondrillina* Bubák & Syd., which successfully controlled rush skeleton weed (*Chondrilla juncea* L.) in Australia (4) and the United States (13), did not infect nontarget plants in greenhouse tests. However, other rusts, more recently considered to be good candidates for weed control, infected nontarget plants in the greenhouse (3,9,10,15). Evidence suggests that most infections on nontarget plants will not be significant in nature (2,9,14), but such greenhouse host-range data is difficult for regulatory agencies to interpret. Greenhouse conditions are optimum for plant growth, and climatic stresses and inter- and intraspecific competition do not influence plant vigor as they do under natural conditions. Greenhouse conditions are also optimum for the pathogen, which makes it difficult to evaluate the efficiency of a biocontrol agent and the actual risk to nontarget plants (14).

The objective of this study was to find measures of disease severity that are biologically meaningful and useful for comparison of rust damage on target and nontarget hosts. In the following experiments, each of five isolates of *Puccinia jaceae* Otth was inoculated both onto the host from which it was collected and onto a nontarget host, cornflower (*Centaurea cyanus* L.), which is susceptible to all five isolates.

MATERIALS AND METHODS

Rust fungi. Research was done in a containment greenhouse under a permit from the USDA Animal and Plant Health Inspection Service. *P. jaceae* is a microcyclic, autoecious rust of *Centaurea* spp. Five isolates of *P. jaceae* were collected in Turkey, Greece, or Bulgaria from 1980 to 1984 for evaluation as biocontrol agents of yellow starthistle, purple starthistle, and diffuse knapweed (Table 1). Each isolate strongly infected the species from which it was collected, and all isolates infected cornflower under greenhouse conditions (Bennett, Cavin, Shishkoff, *unpublished;* 3). For comparison, an isolate of skeleton weed rust (*P. chondrillina*) was also included in this study (Table 1). Because the original evaluation of skeleton weed was done at our facilities using similar methodology (5) and because skeleton weed is known to be controlled by *P. chondrillina,* this host-pathogen pair was considered to be a good positive control. The isolates were stored in liquid nitrogen. To stimulate germination, frozen isolates were heated at 42 C for 6 min upon removal from the liquid nitrogen. Fresh spores were refrigerated (4 C) for up to 4 mo and used without heat shock.

Plants. Seeds of purple starthistle, yellow starthistle, and skeleton weed were collected in California from populations of plants in Solano, Yolo, and Placer counties, respectively. Diffuse knapweed seeds were collected in Chelen County, Washington. Cornflower (cv. Blue Boy) was purchased from Park Seed Company, Greenwood, SC. Seeds were sown in clay pots (10

cm in diameter) filled with a pasteurized greenhouse mix of ProMix Bx (Premier Brands, Stamford, CT), soil, peat moss, vermiculite, perlite, and sand (4:2:2:2:1.5, v/v) with fertilizer (10-10-10) and wetting agent added. Seedlings were thinned to one plant per pot.

Comparison of plant growth habit. Ten plants each of yellow starthistle, purple starthistle, and diffuse knapweed were grown from seed, and the average number of leaves per plant was counted weekly, beginning at 4 wk after the seeds were planted and ending at 22 wk, when almost all the yellow starthistle plants were dead. The leaves in the rosette were counted before each plant bolted, and all leaves on the bolted stem were included afterward. Leaves formed on axillary branches, but these were not counted because of their small size (usually less than 3 cm). Dead leaves were counted and removed.

Inoculation and evaluation of plants. Either whole plants (the entire aboveground portion) or single leaves were inoculated. Each whole plant was inoculated with urediniospores (0.5 mg) by using a turntable settling tower to provide a simulated spore shower (7). Under typical conditions, when 25–30 plants were inoculated with 13–15 mg of spores, 61 ± 13 spores per square centimeter were deposited on leaf surfaces (Shishkoff, *unpublished*). Individual leaves of 5- to 6-wk-old plants were inoculated by the application of a spore suspension (25 mg of urediniospores in 80 mL of distilled water plus 0.13% polyoxyethylene sorbitan monolaurate [Tween-20]) to the leaves with a small polyurethane foam sponge. The plants inoculated by either method were placed in a dew chamber for 12–15 h at 20 C. Control plants, whose leaves were treated with distilled water and Tween-20, were not placed in dew chambers unless specified, in order to avoid accidental infection. After a dew period, the plants were incubated in a greenhouse with natural sunlight plus artificial light set to a 16-h photoperiod; the temperature was set to 20–25 C. The plants were monitored for symptom expression.

The dry root weight of plants was generally used as a measure of the effect of rust infection, even though shoot weights were also measured. In experiments with starthistles and other weeds, the effect of infection on shoot biomass was highly variable, making large sample sizes necessary for statistical analysis. The effect of infection on root biomass, in contrast, was reliably repeatable.

Inoculation of whole plants of different ages. One approach used to determine the effects of infection by *P. jaceae* on target and nontarget hosts (or by *P. chondrillina* on skeleton weed) was whole-plant inoculation of plants of different ages to look for differences in pustule number or for effects on root biomass. The entire aboveground portion of each plant was inoculated twice, either at 2 and 3 wk or at 5 and 6 wk after it was planted. Controls were not inoculated. At 9 wk, the roots were washed to remove all soil, separated from the shoot, dried at 30 C for 5 days, and then weighed. Each isolate was inoculated on its host plant and on cornflower. There were six to 10 plants per treatment, and the experiment was repeated at least once for each host-rust pair. Three weeks after inoculation, pustules per leaf were counted on plants inoculated at 5 and 6 wk. The following data were tabulated: average pustules per leaf for the three most heavily infected leaves, average pustules per leaf for all infected leaves, and average pustules over all leaves.

TABLE 1. Isolates of *Puccinia jaceae* used in this study

Isolate	Host plant[a]	Collector	Location	Date
PST62	Purple starthistle	S. Rosenthal	Turkey	6/9/84
PST66	Purple starthistle	S. Rosenthal	Turkey	6/14/84
YST771	Yellow starthistle	S. Rosenthal	Turkey	6/30/84
DK3	Diffuse knapweed	P. Pecora and G. Campobasso	Bulgaria	6/8/80
DK12[b]	Diffuse knapweed	R. Sobhian	Greece	8/5/83
SW1	Skeleton weed	L. Andres	Italy	1975

[a] Plant from which the isolate was collected.
[b] *P. jaceae* was isolated from a double infection with *P. calcitrapae*.

Leaf-number experiments. The second approach involved inoculation of a different number of leaves on each target plant. Five- to 6-wk-old rosettes were selected for uniformity in size and leaf number. The target hosts were inoculated on zero, four, six, or eight leaves, except for purple starthistle, inoculated with PST66, which was inoculated on zero, two, four, or eight leaves. Cornflower was not inoculated in these trials. After 9 wk, the roots were harvested and dried as previously described. There were seven or eight replicate pots per treatment. The experiments were repeated for each host-rust combination.

Leaf-senescence experiments. The third approach involved inoculation of single leaves to determine the effect of different levels of infection on leaf lifespan. The tests were done on similar 5-wk-old plants with leaves that had been tagged and numbered as they developed. The leaf selected for inoculation on each plant was directly adjacent to a younger leaf that was not fully expanded and to an older leaf of full size. The spore suspension, described above, was further diluted by 1/10 and 1/100, creating high, medium, and low spore concentrations. Approximately one-third of the 15–25 plants were inoculated with each spore concentration, and up to three plants were inoculated with the control solution. The control and inoculated plants were placed in a dew chamber for 12 h, arranged so that they did not touch. The plants were then placed in the greenhouse, and the treated leaves were observed regularly (daily) until they senesced, at which time pustules were counted, and the number of days from inoculation was noted. For most host plants, "leaf senescence" could be arbitrarily defined as the day the petiole lost turgor. Cornflower leaves occasionally died from the tip back, so leaves were considered dead when 90% of the leaf was brittle or had lost turgor. The plants that had been mechanically injured during the experiment were noted and excluded from final data sets. The experiments were repeated two to six times for each host-rust combination. A model for leaf senescence, given in Figure 1, is described by the equation

$$Y = Ae^{-bx} + C$$

where Y is the time in days from inoculation to senescence; x is the number of pustules on the leaf; A is the difference between the lifespan of control (uninoculated) leaves and pustule-saturated leaves; b is the rate of decay; and C is the minimum lifespan

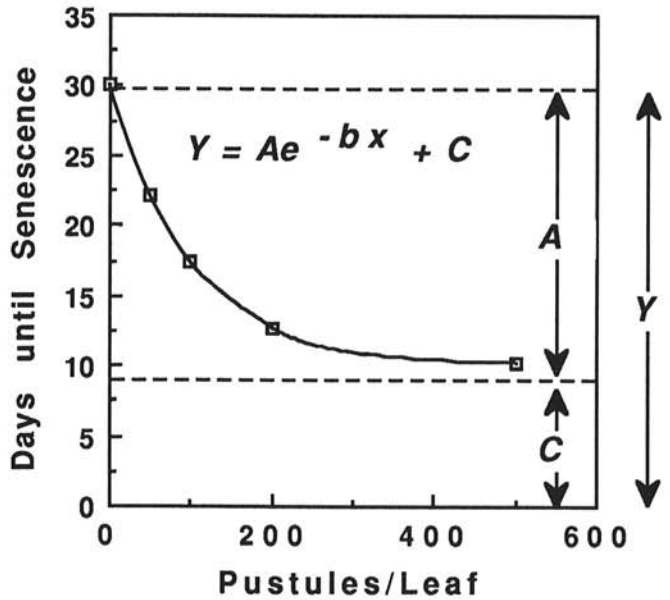

Fig 1. An exponential decay model of leaf senescence, where Y = time in days from inoculation to senescence; x = number of pustules on the leaf; A = the difference between the lifespan of control (uninoculated) leaves and pustule-saturated leaves; b = the rate of decay; C = the minimum lifespan of infected leaves, approached as pustule number approaches infinity; variable D was the y-intercept (the lifespan of control leaves).

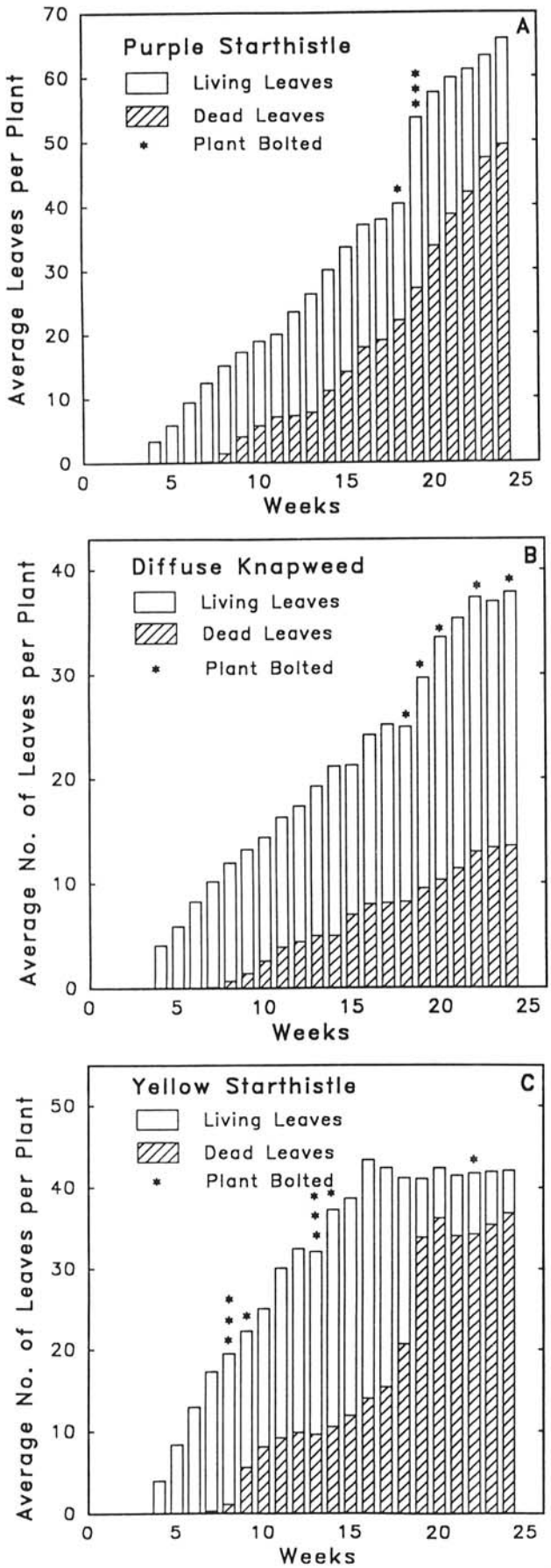

Fig. 2. The number of living and dead leaves on the main axis of three weeds over time.

of infected leaves, approached as pustule number approaches infinity. The lifespan of uninoculated leaves was the y-intercept and was designated *D*.

Statistics. The data were analyzed with SAS mainframe software, version 5.18 (11). The biomass data were tested by analysis of variance or analysis of covariance by using PROC GLM. UNIVARIATE and CORR procedures were used to determine the normality and heterogeneity of residuals for datasets. The data not randomly distributed were transformed using the log plus a constant. All data were tested for replicate effects and replicate by treatment interactions. The leaf-senescence data were subjected to nonlinear regression analysis with PROC NLIN. Either Duncan's multiple range test or least square means test was used for separation of means. Treatments were considered to be significantly different at $P \leq 0.05$.

RESULTS

Comparison of plant growth habit. Purple starthistle plants grown in the greenhouse produced successive waves of short-lived leaves. The average number of living rosette leaves at any particular time was 16, but over the 22 wk that purple starthistle plants were observed, a total of 61 leaves were produced. Less than one-half of the plants had bolted by 22 wk (Fig. 2A). In contrast, most yellow starthistle rosette leaves were alive at the time the plants bolted. This is somewhat obscured in Figure 2C by the fact that yellow starthistle plants bolted irregularly beginning at 4 wk and ending at 22 wk. Nine of 10 yellow starthistle plants had bolted by 8 wk, and only one plant was still alive at 22 wk (Fig. 2C). Diffuse knapweed plants had growth characteristics intermediate between yellow starthistle and purple starthistle plants; they had persistent leaves like the yellow starthistle plants but remained in rosettes for a longer period of time before they bolted (Fig. 2B).

Inoculation of whole plants of different ages. The host-isolate combinations with the highest pustule counts, which were assessed by using any one of the three methods for rating disease (Table 2), were purple starthistle plants infected with PST62 and diffuse knapweed plants infected with DK3. These counts differed significantly only from the combinations with the lowest counts, which were found on cornflower.

The roots of yellow starthistle and skeleton weed plants, inoculated at 2 and 3 wk or at 5 and 6 wk, weighed significantly less than roots from control plants. For other host-isolate combinations, no significant differences occurred among treatments. There was no significant reduction in root biomass of cornflower inoculated with any isolate, but there was a trend toward lower root biomass for plants inoculated at 5 and 6 wk (Table 3).

Leaf-number experiments. The root biomass was significantly reduced when YST71, DK3, DK12, and SW1 were inoculated on four or more leaves of their target hosts. There was no significant reduction in purple starthistle biomass when up to eight leaves were inoculated with PST66 or PST62 (Fig. 3).

Leaf-senescence experiments. In all trials, the number of pustules per leaf was proportional to the rate of leaf senescence (Fig. 4). No leaves, however severely infected, died before pustules broke through the leaf epidermis.

Most of the host-isolate data sets (32 of 40) converged using the SAS procedure PROC NLIN, i.e., a curve could be fit to the data. The eight data sets that did not converge were not used.

The lifespan of uninfected control leaves (variable *D*) differed significantly by host plant; it was greatest for diffuse knapweed plants and least for purple starthistle plants. The reduction in leaf lifespan due to infection (variable *A*) was greatest for diffuse knapweed plants (both isolates) and lowest for purple starthistle plants (both isolates). Healthy purple starthistle leaves did not live very long (27.4–28.5 days), and even severe rust infections shortened leaf lifespan by an average of only 9.3–11.8 days (33–43%). In tests in which yellow starthistle leaves were inoculated, healthy control leaves lived 44.6 days, and heavy infection caused a lifespan reduction of 25 days (57%). Diffuse

TABLE 2. Pustule counts from target hosts (yellow starthistle, purple starthistle, diffuse knapweed, and skeleton weed) or a nontarget host (cornflower) inoculated with isolates of *Puccinia jaceae* or *P. chondrillina*

	Pustule counts[a,b]					
	Host plant			Cornflower		
Isolate	PUST(A)	PUST(B)	PUST(C)	PUST(A)	PUST(B)	PUST(C)
YST71	97 ± 119	59 ± 68	29 ± 39	52 ± 17[c,d]	24 ± 8[c,d]	19 ± 7[c,d]
PST66	116 ± 55	70 ± 36	46 ± 28	52 ± 27[c,d]	25 ± 12[c,d]	21 ± 11[c,d]
PST62	200 ± 78	112 ± 42	69 ± 25	56 ± 32[c]	25 ± 18[c,d]	23 ± 18[d]
DK3	169 ± 71	109 ± 48	59 ± 30	44 ± 21[c,d]	19 ± 10[c,d]	18 ± 11[c,d]
DK12	109 ± 88	66 ± 47	39 ± 31	72 ± 31	37 ± 17[c,d]	32 ± 15
SW1	96 ± 41	45 ± 16	39 ± 16	ND[e]	ND	ND

[a] PUST(A) = Average pustules per leaf and standard deviation for the three most infected leaves; PUST(B) = average pustules per leaf and standard deviation for all infected leaves; PUST(C) = average pustules per leaf and standard deviation for all leaves.
[b] Each isolate was inoculated onto the host from which it was isolated (YST71 onto yellow starthistle, PST62 and PST66 onto purple starthistle, DK3 and DK12 onto diffuse knapweed, and SW1 onto skeleton weed). All isolates infect cornflower except SW1 (*P. chondrillina*).
[c] Pustule counts differ significantly from counts of the host-rust combination PST-PST62.
[d] Pustule counts differ significantly from counts of the host-rust combination diffuse knapweed-DK3.
[e] Not done.

TABLE 3. Root biomass of *Centaurea* spp. or cornflower plants inoculated with five isolates of *Puccinia jaceae* compared to biomass of uninoculated controls

Treatment[a]	Dry root weight (g)					
Weeks	YST71	PST66	PST62	DK3	DK12	SW1
Host plants[b]						
0	2.32	2.89	1.32	1.58	0.36	1.52
2 and 3	1.77[c]	2.54	1.24	1.58	0.27	1.25[c]
5 and 6	1.70[c]	2.82	1.23	1.35	0.34	0.84[c]
Cornflower						
0	0.29	0.29	0.26	1.14	0.23	...
2 and 3	0.24	0.28	0.21	1.06	0.20	...
5 and 6	0.23	0.21	0.20	0.99	0.17	...

[a] Plants were inoculated twice, either at 2 and 3 wk or at 5 and 6 wk after they were planted, and compared to uninoculated controls (0 wk).
[b] Each isolate was inoculated onto the host from which it was isolated (YST71 onto yellow starthistle, PST62 and PST66 onto purple starthistle, DK3 and DK12 onto diffuse knapweed). All isolates infect cornflower except SW1 (*P. chondrillina*).
[c] Root biomass differed significantly from that of control plants.

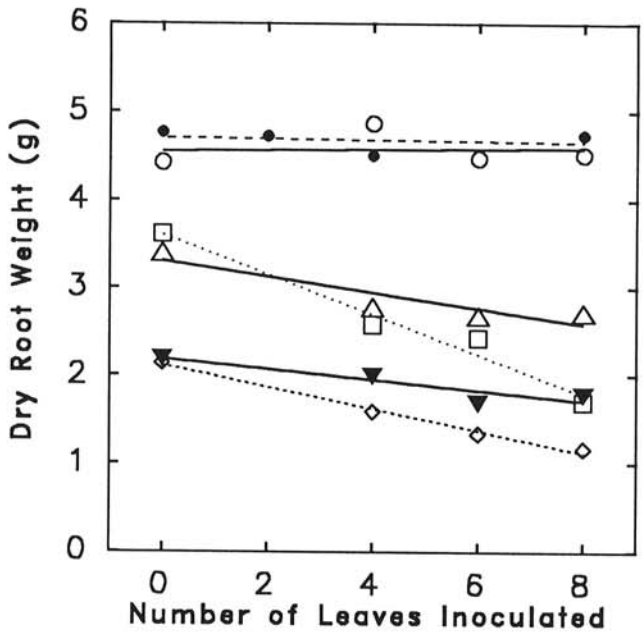

Fig. 3. The root biomass of target weeds inoculated on increasing numbers of leaves with their respective isolates of *Puccinia jaceae* or *P. chondrillina*. Regression lines for purple starthistle had a slope that did not significantly differ from zero ($P = 0.05$). ● = PST66 on purple starthistle; ○ = PST62 on purple starthistle; □ = YST71 on yellow starthistle; △ = DK3 on diffuse knapweed; ▼ = DK12 on diffuse knapweed; ◇ = SK1 on skeleton-weed.

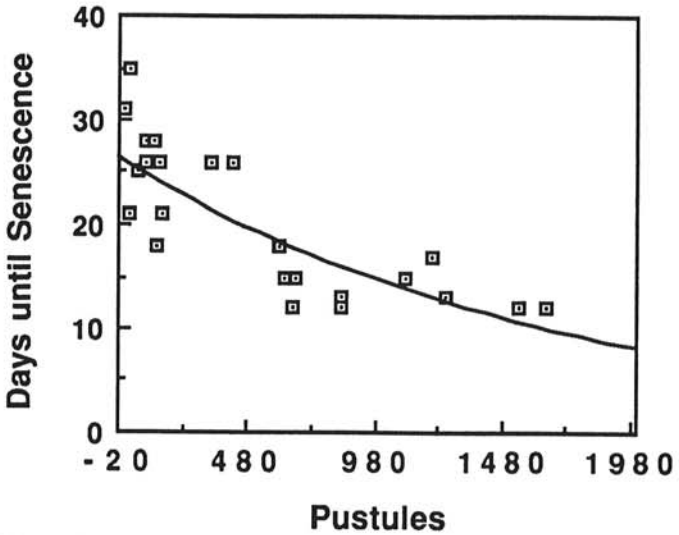

Fig. 4. Lifespan (days) of yellow starthistle leaves infected with increasing numbers of pustules of *Puccinia jaceae* isolate YST71.

knapweed leaves were persistent, with a lifespan of 85–103 days. Heavy infection caused a lifespan reduction of 66–76%. In tests in which skeleton weed leaves were inoculated, healthy controls lived 48.3 days, and heavy infection caused a lifespan reduction of 67% (Table 4).

For target hosts, variables *b* and *C* did not differ significantly among treatments. The rate of leaf senescence relative to pustule number (variable *b*) did not differ significantly by treatment but varied considerably among replicates (0.001–1.50). The minimum lifespan of infected leaves, 15.6–29.5 days (variable *C*), did not differ significantly among treatments (Table 4).

When cornflower-*P. jaceae* inoculations were compared for variables *A, b, C,* and *D,* there were no significant differences (Table 4).

DISCUSSION

Our objective was to find measures that provided both rapid evaluation of pathogens for biocontrol of weeds and realistic evaluation of risk for nontarget hosts.

Mortensen (8) described a useful system for evaluating rust disease severity using a scale of 0–9 for percentage of leaf area affected. This system attempts to establish criteria for field testing and depends in part on the degree of infection on the nontarget plant. However, for our isolates, there was no correlation between disease severity measured as pustule counts and root biomass reduction. The root biomass was reduced when yellow starthistle plants were inoculated with *P. jaceae* isolate YST71 and when skeleton weed plants were inoculated with *P. chondrillina,* despite

the fact that inoculation caused only moderate pustule numbers that were not significantly different from other host-isolate combinations. Purple starthistle plants inoculated with PST66 and PST62 sustained relatively high pustule numbers without reduced root biomass. We suggest that an assessment of disease severity based on pustule counts can be a weak indicator of biocontrol potential. Schtienberg (12) found that visual estimates of foliar disease did not accurately predict the magnitude of the reduction of transpiration caused by infection. For example, at low levels of rust infection, transpiration was higher in infected plants than in healthy plants; and at higher levels of disease, the reduction in transpiration was smaller than would be expected for the leaf area affected (12).

A direct measure of a plant's reproductive potential is seed number or quality. In field tests, Baudoin et al (2) found a significant reduction in seed number and quality in musk thistle plants infected with *P. carduorum* Jacky. However, seed characteristics are difficult to measure in outcrossing plants grown under greenhouse conditions. An indirect measure, such as the biomass of the primary storage organ, in this case the root system, may be a satisfactory indicator in the greenhouse.

Although isolates affected each target weed differently, they affected cornflower the same way; i.e., cornflower plants inoculated with YST71 reacted no differently than those inoculated with diffuse knapweed or purple starthistle isolates, even though YST71 significantly reduced root biomass on yellow starthistle plants.

Host characteristics might be used to explain these differences in target-plant response to infection. Purple starthistle plants produced many short-lived leaves that were constantly replaced, whether or not they were infected. As a biennial, purple starthistle can remain in rosette form for 2 yr or more, until it has accumulated enough energy to bolt. Diffuse knapweed plants had long-lived leaves, and the impact of infection was greater as determined by loss of root biomass. Diffuse knapweed is also a biennial, and does not bolt until conditions are right. Yellow starthistle plants produce a determinant number of leaves that are not replaced; therefore, the effect of infection is not mitigated by new growth before the plant bolts at the end of the season.

TABLE 4. Regression variables from the equation $Y = Ae^{-bx} + C$ used in analyses of variance to compare the effect of infection of target host leaves by isolates of *Puccinia jaceae* or *P. chondrillina* or infection of cornflower by *P. jaceae*

	Regression variables[a]			
	A	*b*	*C*	*D*
Target host-isolate[b]				
DK-DK3	73.4 a	0.0043 a	29.5 a	103.0 a
DK-DK12	55.9 a	0.0036 a	29.2 a	85.2 a
SW-SW1	32.6 b	0.0057 a	15.7 a	48.3 b
YST-YST71	25.2 bc	0.0829 a	19.4 a	44.6 b
PST-PST62	11.8 dc	0.0035 a	15.6 a	27.4 c
PST-PST66	9.3 d	0.2967 a	19.2 a	28.5 c
Cornflower-isolate				
CF-DK3	53.0 a	0.0094 a	33.5 a	86.5 a
CF-DK12	49.8 a	0.0167 a	69.8 a	119.7 a
CF-YST71	64.6 a	0.0070 a	36.3 a	100.9 a
CF-PST62	52.1 a	0.0111 a	27.1 a	79.1 a
CF-PST66	75.7 a	0.0078 a	37.6 a	113.2 a

[a]An analysis of variance was done on regression variables for leaf senescence curves derived when each isolate was inoculated on its target host. A separate analysis of variance was done on variables for curves derived when each rust isolate was inoculated onto cornflower. The variables come from the exponential decay model described in the text. A = The reduction in leaf lifespan due to infection (days); b = a measure of the steepness of decay (per pustule); C = the minimum lifespan of infected leaves (approached as pustule number approaches infinity); D = the y-intercept, the lifespan of control leaves (days). Values in a column followed by the same letter are not significantly different at $P = 0.05$, according to the least square means test.

[b]Each isolate was inoculated onto the host from which it was isolated or onto cornflower. DK = Diffuse knapweed; SW = skeleton weed; YST = yellow starthistle; PST = purple starthistle; CF = cornflower.

Yellow starthistle appeared to be the most sensitive to reduction of root biomass in these studies. Although skeleton weed is a perennial, each season the plant produces a rosette of leaves much the way yellow starthistle does. The effect of a rust on root biomass, therefore, seems to depend on the relative value of each leaf to the plant.

It was hoped that senescence curves for each host-isolate would yield an estimate of the "biologically significant" amount of disease by allowing one to determine the number of pustules at some point of physiological significance. For instance, a significant pustule number might be found at the inflection point at which increasing pustule number no longer reduced leaf lifespan dramatically. However, variable *b* did not differ among treatments and varied considerably from trial to trial within a treatment, leading one to suspect the "biologically significant" amount of disease is strongly influenced by environmental parameters such as temperature or soil water potential.

In the future it may be possible to predict how characteristics of a weed's growth will influence its suitability as a target for biological control. Perhaps plants with short-lived and constantly replaced leaves will be able to outgrow a rust infection, especially where epidemic development is not likely to keep pace with production of new plant tissue. Perhaps infected biennials will be able to delay reproduction by remaining in a rosette stage until conditions are unfavorable for the rust. These answers will come through coordinated greenhouse and field research.

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