A Rapid Bioassay for the Assessment of Pathogenic Variation in *Colletotrichum gloeosporioides* infecting *Stylosanthes scabra*

SUKUMAR CHAKRABORTY and PETER N. JONES, Division of Tropical Crops and Pastures and Institute of Plant Production and Processing Biometrics Unit, CSIRO, 306 Carmody Road, St Lucia, Queensland 4067, Australia

ABSTRACT

Chakraborty, S., and Jones, P. N. 1993. A rapid bioassay for the assessment of pathogenic variation in *Colletotrichum gloeosporioides* infecting *Stylosanthes scabra.* Plant Dis. 77:1016-1020.

Pathogenic variation in *Colletotrichum gloeosporioides,* causing anthracnose disease of the tropical pasture legume *Stylosanthes scabra,* was studied using 3-day-old germlings and 6-wk-old seedlings of six host differentials. Disease expression of the host differentials in germling stage was optimized by standardizing pre- and postinoculation temperatures. To test the effectiveness of the germling assay, 10 field isolates were assayed with both germling and seedling assays. There was a significant correlation ($r = 0.77$) between the two assays. Despite significant variation among runs in both assays, the germling assay correctly assigned 11 of the 12 isolates to the same races as did the traditional seedling assay. Compared to the seedling assay, the germling assay is rapid, uses less inoculum and space, and can be carried out under controlled environmental conditions.

Anthracnose disease, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz., seriously limits the utilization of pasture legumes of the genus *Stylosanthes* in tropical and subtropical Australia. In the 1970s, 500,000 ha of improved pastures with susceptible cultivars of *Stylosanthes humilis* Kunth were devastated by this disease. Over the past two decades, rapid adaptation by the pathogen to new cultivars has meant that seven of the 12 cultivars released in Australia are no longer in commercial use (2,8). Anthracnose causes reductions in dry matter and seed yields of moderately resistant cultivars of *Stylosanthes hamata* (L.) Taub. currently in use.

In Australia, the fungus shows a high degree of pathogenic variation, with two distinct types varying in host range, conidial morphology, and symptomatology (9). Type A produces conidia rounded at both ends and causes discrete lesions on aerial plant parts on most species of *Stylosanthes.* Type B produces bullet-shaped conidia and causes blight of the terminal shoots in a few species, including *Stylosanthes guianensis* (Aubl.) Sw., which includes commercial cultivars adapted to specific geographical regions. Type A is economically more important, because it infects all the widely adapted commercial cultivars of *Stylosanthes scabra* Vogel and *S. hamata* currently grown in Australia. At least four different races have been reported within each type in Australia (10).

Accepted for publication 31 May 1993.

© 1993 The American Phytopathological Society

We investigated the use of genotype mixtures (5) and forms of partial resistance (4) for the long-term management of this pathogen in perennial *Stylosanthes*-based pastures. The rate at which the pathogen adapts to the host, the relative fitness of these new pathotypes, and the nature and direction of genetic shift in the host population as a result of outcrossing will be key determinants of the durability of these management strategies. To quantify, analyze, and predict changes in the pathogen population, a large number of field isolates needs to be assessed. The current method for detecting pathogenic variation involves the inoculation of 6–8 wk old seedlings of a host differential set with a conidial suspension of the pathogen, and subsequent assessment for disease severity 10 days after inoculation (6). The procedure takes 8–10 wk to complete. This method is time-consuming and requires a great deal of glasshouse space to test more than a few isolates. The method gives variable results because of, among other factors, fluctuations in a glasshouse environment between runs. To develop a rapid bioassay for the routine analysis of pathogenic variation in field isolates of *C. gloeosporioides,* we compared disease reactions of the host differentials in their cotyledon (germling) and seedling stages. We optimized the disease reactions of the germlings by standardizing pre- and postinoculation temperatures and compared the effectiveness of the germling assay by using 10 pathogen isolates.

MATERIALS AND METHODS

Seedling and germling assays. Accessions 36260, 55860, Q10042, and 93116 from the Australian Tropical Forage Genetic Resource Centre, Division of Tropical Crops and Pastures, CSIRO, and cultivars Fitzroy and Seca of *S. scabra* were used as host differentials (6). Of these, Fitzroy is susceptible and 93116 is resistant to all races. Seca is resistant to all races except 3, 36260 and Q10042 are susceptible to races 4 and 4a, and 55860 is partially resistant to all races (3). Seeds from single plants of the accessions grown in a glasshouse were used. For the seedling assay, seeds were mechanically scarified with sandpaper, germinated in petri dish moist chambers, and sown singly in 4 × 4 cm (65 ml) peat cups in riverbank soil. Accessions were arranged at random in a plastic tray with 30 peat cups to give five replicate seedlings for each accession/cultivar. Seedlings were grown for 6–8 wk in a naturally illuminated glasshouse (14 ± 0.5 hr of light per day at 32 ± 5 C with 40–60% RH, and at night 23 ± 3 C with 80–90% RH).

Seeds for the germling assay were similarly scarified, surface sterilized in sodium hypochlorite (1% available chlorine) for 5 min, then rinsed twice with sterile distilled water and blotted dry on a sterile filter paper. Five or six seeds of an accession were placed in each cell of a square petri dish divided into 25 compartments (Replidish, Sterilin Limited, Feltham, England) and soaked in 0.5 ml of sterile distilled water. Accessions were placed in the compartments at random, and four replicates were maintained for each accession. Each Replidish, without its lid, was placed inside a clear plastic box and covered with a clear plastic lid. A thin layer of distilled water in the box provided high RH. Boxes were incubated under ambient conditions (13–14 hr day at 28 ± 1 C, and at night 21 ± 1 C), and seeds produced a carpet of germlings in 3–4 days.

Initially, the germling and seedling assays were compared by using three isolates of *C. gloeosporioides*: SR 24, WRS 20, and WRS 32 representing races 3, 4, and 4a, respectively (3). Cultures were grown on oatmeal agar, and inocula containing 10^6 conidia per milliliter were prepared by methods described previously (6). Both seedlings and germlings were inoculated by spraying to runoff. Following inoculation, seedlings were incubated in the darkness for 48 hr at 25 C and then returned to the glasshouse. Germlings were incubated under ambient

laboratory conditions (AMB) throughout.

Seedlings were assessed 10 days after inoculation on a 10-point scale (0 = no visible symptoms, 1 = 1–3% of leaf area diseased, 2 = 4–6%, 3 = 7–12%, 4 = 13–25%, 5 = 26–50%, 6 = 51–75%, 7 = 76–87%, 8 = 88–94%, and 9 = 95–100%) (3). Germlings were assessed 2–3 days after inoculation on a five-point scale (0 = both cotyledons and hypocotyl green or white and healthy; 1 = cotyledon pale brown and water soaked, hypocotyl green or white and healthy; 2 = cotyledon brown and water soaked, hypocotyl pale brown; 3 = cotyledon dark brown or black, often rotting, hypocotyl dark brown or black; and 4 = cotyledon and hypocotyl completely rotted and shriveled). The experiment was repeated once, and Spearman's rank correlation (13) was used to test the similarity of the two assays.

Pre- and postinoculation temperature effects on germling disease severity. Three controlled environment cabinets (CECs) and AMB were used to impose four pre- and postinoculation treatments in a factorial design of four treatments by two times. The first CEC was maintained at a 14-hr photoperiod with 30 ± 0.5 C during the day and 25 ± 0.5 C at night (30/25), the second at a 14-hr photoperiod with 25 ± 0.5 C for both day and night (25DN), and the third at 25 ± 0.5 C in darkness (25D). AMB was as described previously. Germlings, grown in the four environments for 3–4 days, were inoculated with 10^6 conidia per milliliter of isolate SR 24 and incubated in each of the four environments. The experiment was repeated once, and data were analyzed by the analysis of variance techniques of the Genstat 5 statistical software package (12).

Comparison of seedling and germling assays. Ten isolates of *C. gloeosporioides* collected from *S. scabra* pastures in Queensland, including one (WRS 32) used in the preliminary comparison, were used. Except for the 25DN CEC used for growing germlings both before and after inoculation, the methods used for raising seedlings, inoculation, and disease assessment, and the number of replicates used were as described previously.

Table 1. Isolates of *Colletotrichum gloeosporioides* used in the present study with their race designations made by *Stylosanthes scabra* differentials in their cotyledon (germling) and seedling stages

Isolate	Race assignment: Seedlings	Germlings
CS 216	4a	4
CS 235	4a	4a
CS 255	1	4a
CS 306	4a	4a
CS 330	1	1
CS 333	1	1
CS 334	3	3
CS 336	4a	4a
CS 348	4	4a
SR 24[y]	3	3
WRS 20[y]	4	4
WRS 32[z]	4a,4	4,4

[y] Used in preliminary comparison only.
[z] Used both in the preliminary test and as one of the 12 isolates used to compare the two assays.

RESULTS

Seedling and germling assays. *C. gloeosporioides* produced general water-soaked lesions on germlings, often covering the entire hypocotyl and cotyledons. Accession 93116, which has a high level of resistance against all known races, both as a seedling and as an adult plant

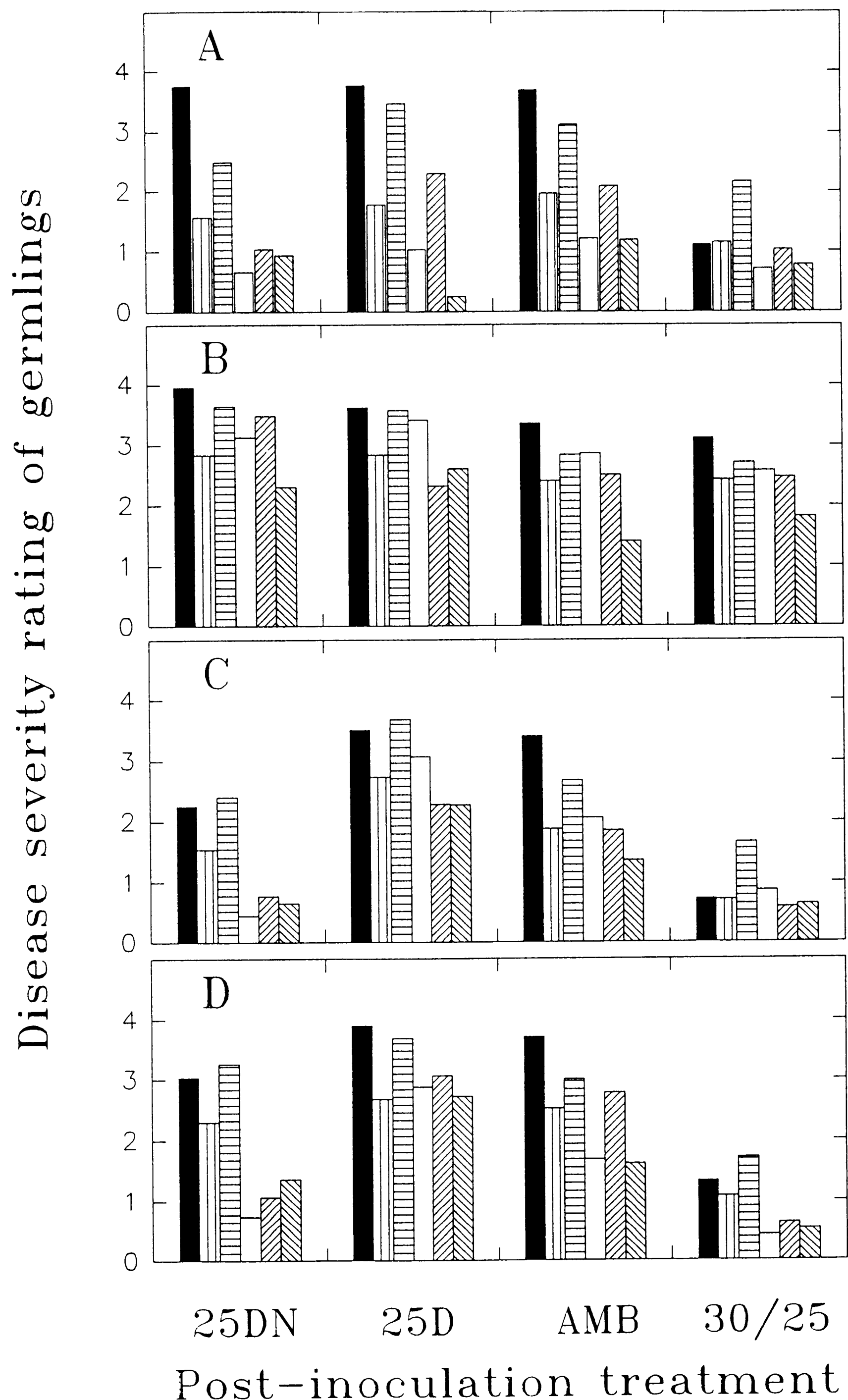

Fig. 1. Anthracnose severity of *Stylosanthes scabra* accessions 93116 (▧), 55860 (▨), 36260 (▤), and Q10042 (□), and cultivars Seca (▥) and Fitzroy (■) inoculated with race 3 of *Colletotrichum gloeosporioides*. Seedlings in their cotyledon stage (germlings) were grown at a given temperature, inoculated, and then exposed to 25 C day and night (25DN), 25 C in the darkness (25D), ambient laboratory conditions (AMB), or 30 C day and 25 C night (30/25). **(A)** Germlings grown at 25 C day and night before inoculation, **(B)** germlings grown at 25 C in the darkness before inoculation, **(C)** germlings grown at ambient laboratory conditions before inoculation, and **(D)** germlings grown at 30 C day and 25 C night before inoculation. Standard error of difference between means is 0.472.

(3), showed some lesion development as a germling. Using a mean disease rating of greater than 2.5 for seedling and greater than 1.5 for germling to denote susceptibility, both assays assigned isolates SR 24 to race 3 and WRS 20 to race 4. Isolate WRS 32, assigned to race 4a by the seedling assay, was assigned to race 4 in the germling assay (Table 1). Spearman's rank correlation coefficients for disease ratings of host differentials between the two assays was 0.89 (significant at $P < 0.05$) for SR 24, 0.74 (not significant, $P > 0.05$) for WRS 20, and 0.76 (significant at $P < 0.05$) for WRS 32.

Pre- and postinoculation temperature effects on germling disease severity. Overall, plants maintained at the 25D CEC before (mean severity rating 2.8) or after inoculation (mean rating 2.8) developed significantly ($P < 0.05$) higher disease severities than any other pre- (mean rating 1.8–2.1) or postinoculation (mean rating 1.3–2.4) treatments. A postinoculation exposure to 30/25, on the other hand, caused a significant decrease in severity, with up to 57% reduction for the highly susceptible Fitzroy (Fig. 1). The host differentials reacted differently to the pre- and postinoculation treatments, giving a significant ($P < 0.05$) three-way pre × post × differential interaction. The two-way interactions between pre- or post- and host differential also were significant. Overall, there was no significant difference in severity between AMB and 25DN applied as a preinoculation treatment; however, germlings exposed to AMB after inoculation produced significantly higher severities than those at 25DN. Based on these results, a pre- and postinoculation environment of 25DN was considered optimum and was used in all subsequent studies.

Comparison of seedling and germling assays. There was good agreement between the two assays with an overall Pearson correlation coefficient of 0.77, which was highly significant ($P < 0.0001$); and the correlation coefficient for individual isolates ranged from 0.59 to 0.97. A plot of the two assays over the 10 isolates showed that they were linearly related (Fig. 2). Of the 10 isolates tested, seven were correctly assigned to their respective races by using data from both seedling and germling assays (Table 1). Data on disease severity for germlings and seedlings were treated separately for variance component analysis and combined for comparison purposes by analysis of variance.

In the combined analysis, severity ratings were converted to normal scores to allow scores measured over different ranges to be compared (11). In this analysis, differentials Fitzroy and 55860, with low to moderate resistance to most of the 10 isolates, had similar disease severities as both germlings and seedlings (Table 2). In all other differentials except Q10042, severity levels on germlings were significantly ($P < 0.05$) higher than those on seedlings; while in Q10042, seedlings showed significantly ($P < 0.05$) higher severities than germlings. This resulted in a significant ($P < 0.05$) assay × differential interaction in the combined analysis.

When analyzed separately, the germling and seedling assays gave coefficients of variation (CV) of 40.1 and 45.5%, respectively. In both assays, the biggest contributors to the variance components were the interaction terms involving the number of times an assay was repeated (runs) and the final error term (Table 3). The CV was estimated for increasing numbers of replicates within an assay as well as for increasing numbers of runs. For both assays, the CV was only marginally improved by increasing the number of replicates within an assay; on the other hand, the CV was progressively reduced with an increase in the number of runs (Fig. 3). To reduce the CV to 20%, it would be necessary to repeat the germling and seedling assays 8 and 10.3 times, respectively, when four replicates within a run were used.

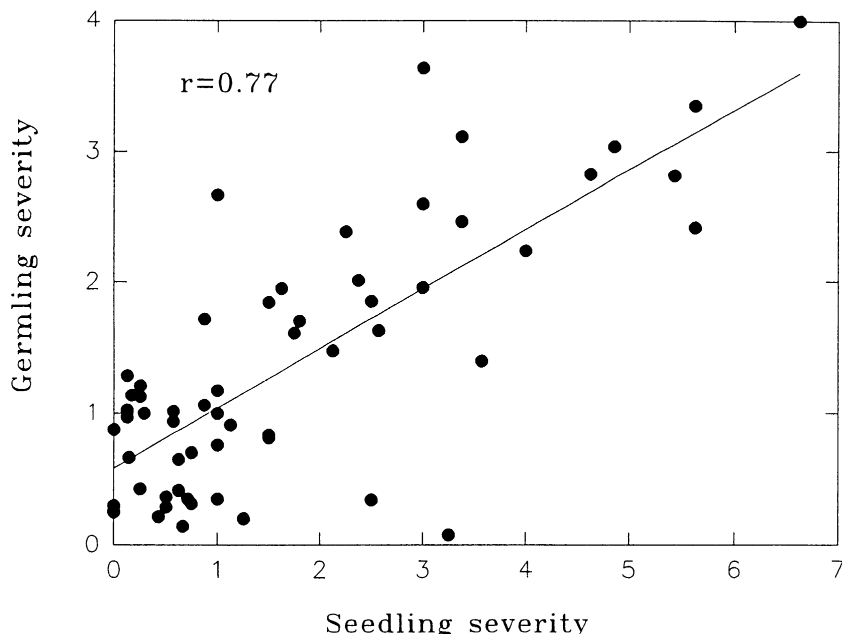

Fig. 2. Correlation among anthracnose severities of six differentials of *Stylosanthes scabra* in their cotyledon (germling) and seedling stages.

DISCUSSION

We report on the development of a rapid protocol for detecting pathogenic variation in *C. gloeosporioides* infecting *S. scabra.* The germling assay presents a number of advantages over the conventional seedling assay: it is rapid (7–8 days compared to 8–10 wk); uses less inoculum (10 ml for germlings vs. 200 ml or more for seedlings) to enable the testing of weakly sporulating isolates; and is low in cost and use of space, which enables the bioassay to be carried out under reproducible controlled environmental conditions.

Of 12 isolates (3 + 10, with one common isolate) used to compare the two assays, four were allocated to different races by the two assays. Of these, three (one in the preliminary test and two of the 12 in the final comparison) involved misallocations between races 4 and 4a. WRS 32, which was classified as race 4a by seedling assay in the preliminary trial, was classified race 4 in both assays in later experiments (Table 1). This is similar to previous studies, where the distinction between races 4 and 4a has not been all that clear with the seedling assay (3). This may reflect the level of heterogeneity in accessions 36260 and Q10042. The only serious mismatch was that of CS 255, which was classified as race 1 in the seedling and race 4a in the germling assay. The germling assay assigned 11 of the 12 isolates to the same races as did the seedling assay. This gives the germling assay a 92% accuracy.

Table 2. Mean anthracnose severities of six host differentials of *Stylosanthes scabra* inoculated with 10 isolates of *Colletotrichum gloeosporioides* in their cotyledon (germling) and seedling stages[y]

Host differential	Seedling severity (normal scores)[z]	Germling severity (normal scores)
55860	−2.121	−2.029
93116	−3.672	−2.165
36260	−1.520	−0.655
Seca	−4.040	−2.069
Fitzroy	−0.349	−0.512
Q10042	−2.117	−2.814

[y] Standard error of difference between means = 0.213.
[z] Standard normal deviate that gives the lower tail probability equal to the severity rating (scaled to lie between 0 and 1).

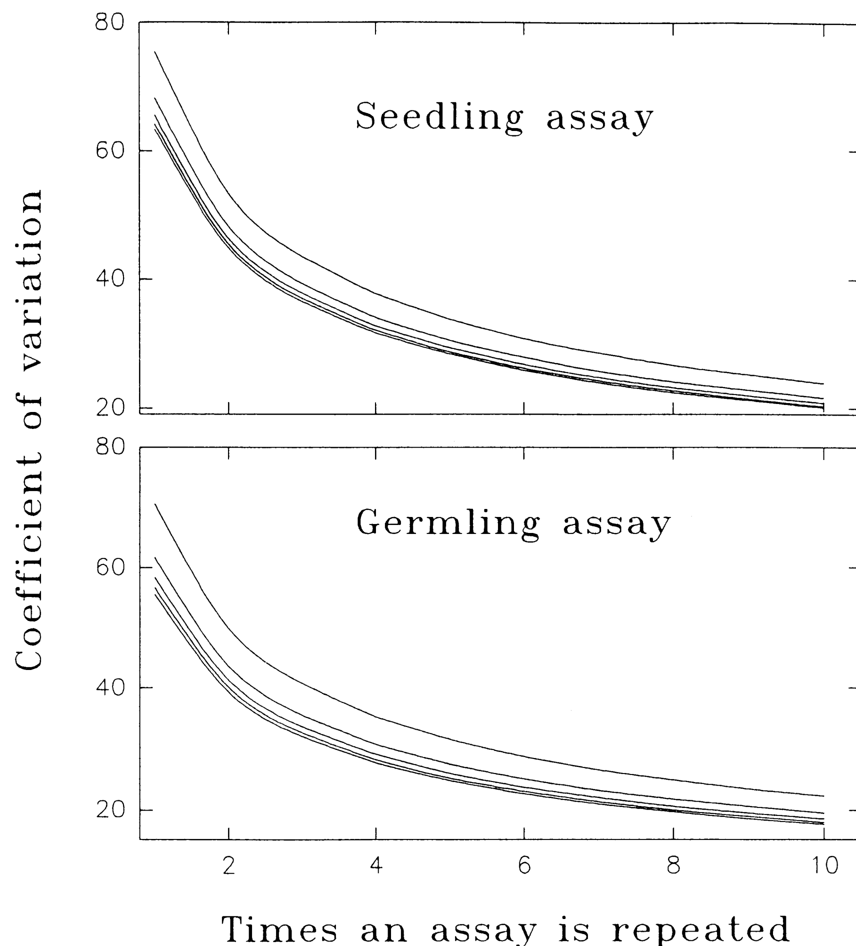

Fig. 3. Relationship between coefficient of variation and the number of times assays were repeated among seedlings and among germlings. The five lines in each graph represent, from top to bottom, one to five internal replications.

Table 3. Analysis of variance and its components for data on anthracnose severity of germlings and seedlings of *Stylosanthes scabra* inoculated with 10 different isolates of *Colletotrichum gloeosporioides*

		Seedling			Germling		
Source	**df**[x]	**Mean square**	***F* ratio (*P*)**[y]	**Variance component**	**Mean square**	***F* ratio (*P*)**	**Variance component**
Replication (run)	1	2.96	*[z]	0.0	0.00	*	0.0
Isolate (iso)	9	11.66	0.75 (0.6)		5.36	0.84 (0.6)	
Differential (dif)	5	195.32	46.5 (0.0001)		62.23	9.8 (0.01)	
Run × iso	9	15.47	6.45 (0.0001)	0.54	6.35	7.0 (0.0001)	0.23
Run × dif	5	4.201	1.75 (0.14)	0.04	6.35	6.99 (0.0001)	0.14
Iso × dif	45	4.79	2.00 (0.01)		2.23	2.46 (0.002)	
Run × iso × dif	45	2.39	3.96 (0.0001)	0.45	0.91	2.09 (0.0001)	0.12
Error	360	0.60		0.60	0.43		0.43

[x] Degrees of freedom.
[y] Figure in parentheses is the probability level for significance.
[z] No exact *F* test can be calculated.

The level of variation in the seedling assay has been and continues to be an area of concern in the evaluation of pathogenic variability in this specialized necrotrophic fungus. Heterogeneity in some accessions in our differential set, variability in single-spore isolates of the pathogen, and changes in glasshouse conditions between assays have been responsible for variations in the seedling assay. By using standardized conditions, we have minimized variations in the environment in the germling assay. It is, however, impractical to consider repeating the germling assay eight times to reduce variability, especially when dealing with a large numbers of isolates. The coefficient of variation in the germling assay, under well-controlled environmental conditions, is still high. Further work needs to be done to reduce variability within isolates and differentials. Measures such as viability and infection efficiency of the isolates may be used as weighting factors in the interpretation of disease-severity patterns between runs. Other measures, including the use of clonally propagated materials or near-isogenic lines to reduce variability within host differentials, may be useful. Until a more reproducible method is found, the germling assay can be used in preference to the traditional seedling assay to study pathogenic variation in *C. gloeosporioides.*

In common with other studies (1,7), significant differences in resistance levels were noted in our results between juvenile and more mature plants. Some isolates can cause moderate to high levels of disease on Seca in its seedling stage, yet this cultivar sustains little damage in the field where these isolates occur (Chakraborty, *unpublished*). Provided caution is used in extrapolating results from tests on juvenile plants to predict reactions of adult plants, the germling assay is suitable for monitoring changes in pathogen populations under different management strategies. Being contained and controlled, it may also be used for screening exotic *C. gloeosporioides* isolates when quarantine restrictions may not allow testing under glasshouse or field environments.

ACKNOWLEDGMENTS

We thank Doug Ratcliff for his assistance in the statistical analysis and Edward Liew and Agnieszka Poplawski for technical assistance.

LITERATURE CITED

1. Bussey, M. J., and Stevenson, W. R. 1991. A leaf disk assay for detecting resistance to early blight caused by *Alternaria solani* in juvenile potato plants. Plant Dis. 75:385-390.
2. Cameron, D. F. 1990. Shrubby stylo (*Stylosanthes scabra*). Plant Var. J. 3:33-35.
3. Chakraborty, S., Cameron, D. F., Irwin, J. A. G., and Edye, L. A. 1988. Quantitatively expressed resistance to anthracnose (*Colletotrichum gloeosporioides*) in *Stylosanthes scabra.* Plant Pathol. 37:529-537.
4. Chakraborty, S., Pettitt, A. N., Boland, R. M., and Cameron, D. F. 1990. Field evaluation of quantitative resistance to anthracnose in *Stylosanthes scabra.* Phytopathology 80:1147-1154.
5. Chakraborty, S., Pettitt, A. N., Cameron, D. F., Irwin, J. A. G., and Davis, R. D. 1991. Anthracnose development in pure and mixed stands of the pasture legume *Stylosanthes scabra.* Phytopathology 81:788-793.
6. Davis, R. D., Irwin, J. A. G., and Cameron, D. F. 1984. Variation in virulence and pathogenic specialization of *Colletotrichum gloeosporioides* isolates from *Stylosanthes scabra* cvv. Fitzroy and Seca. Aust. J. Agric. Res. 35:653-662.
7. Eenink, A. H., and Dejong, C. J. 1982. Partial resistance in lettuce downy mildew (*Bremia lactucae*). 3. Correspondence between resistance levels of cotyledons and leaf discs and resistance of adult plants. Euphytica 31:761-770.
8. Eyles, A. G. 1989. Forage cultivars released for use in Queensland. Trop. Grassl. 23:115-117.
9. Irwin, J. A. G., and Cameron, D. F. 1978. Two diseases of *Stylosanthes* spp. caused by *Colletotrichum gloeosporioides* in Australia, and pathogenic specialization within one of the causal organisms. Aust. J. Agric. Res. 29:305-317.
10. Irwin, J. A. G., Cameron, D. F., and Lenne, J. M. 1986. Anthracnose problems with *Stylosanthes.* Pages 38-46 in: Proc. Aust. Conf. Trop. Pastures, 3rd. G. J. Murtagh and R. M. Jones,

eds. Tropical Grassland Society of Australia, Occ. Publ. No. 3.
11. Kotz, S., and Johnson, N. L. 1985. Encyclopedia of Statistical Sciences. Vol. 6, Wiley-Interscience Publications. John Wiley & Sons, New York.
12. Payne, R. W., Lane, P. W., Ainsley, A. E., Bicknell, K. E., Digby, P. G. N., Harding, S. A., Leech, P. K., Simpson, H. R., Todd, A. D., Verrier, P. J., White, R. P., Gower, J. C., and Wilson, G. T. 1987. Genstat 5 Reference Manual. Clarendon Press, Oxford.
13. Steel, R. G. D., and Torrie, J. H. 1986. Principles and Procedures of Statistics. A Biometrical Approach. 2nd ed. McGraw-Hill, Singapore.