Differentiation of *Ustilago scitaminea* Isolates in Greenhouse Tests

A. G. GILLASPIE, JR., Research Plant Pathologist, and R. G. MOCK, Plant Pathologist, Beltsville Agricultural Research Center, ARS, USDA, Beltsville, MD 20705, and J. L. DEAN, Research Plant Pathologist, U.S. Sugarcane Field Station, ARS, USDA, Canal Point, FL 33438

ABSTRACT

Gillaspie, A. G., Jr., Mock, R. G., and Dean, J. L. 1983. Differentiation of *Ustilago scitaminea* isolates in greenhouse tests. Plant Disease 67:373-375.

Seven sugarcane (*Saccharum* interspecific hybrids) clones were inoculated in a containment greenhouse by hypodermic injection with *Ustilago scitaminea* teliospores obtained from Argentina, Florida, Hawaii, Taiwan, and Zimbabwe. Seven months after inoculation, six different isolates (races) could be differentiated on five of the clones under greenhouse conditions.

Smut of sugarcane (*Saccharum* interspecific hybrids) caused by *Ustilago scitaminea* H. & P. Syd. decreases the yield and quality of infected plants (8,16). The disease is characterized by a long unbranched whiplike structure that develops from the plant apex. The whip consists of a hard core of parenchyma and fibrovascular elements surrounded by masses of dark-colored spores encased in a thin silvery membranous sheath (1,11).

Sugarcane smut, first discovered in Natal, South Africa, in 1877 (14), was believed to be confined to the Eastern Hemisphere until it was found in Argentina in 1940 (1). The disease was first reported on the United States mainland in Florida in 1978 (17) and in Louisiana (9) and Texas (19) in 1981. The disease has been severe in nearly all sugarcane-growing areas of the world at one time or another (5) and can be sufficiently severe to threaten the agricultural economy of an area (1). Although several methods of control are available, the most satisfactory is use of smut-resistant clones (1,5).

In recent years, a number of clone/smut evaluations have been undertaken around the world with widely varying results (20). Factors responsible for the varying results include the presence of different pathogenic races, variation in clones due to location, and different inoculation techniques (1,7,11,20). Races have been reported in Hawaii (3), Taiwan (10), and Brazil (5).

The purpose of this research was to compare the pathogenicities of *U. scitaminea* isolates from different parts of the world by using a common environment, one set of sugarcane differentials, and one inoculation method to determine whether races of the fungus can be separated. Similarities and differences in pathogenicity of the various isolates on sugarcane clones used as differentials were noted and evaluated.

MATERIALS AND METHODS

Facility. This experiment was conducted at the Plant Disease Research Laboratory (PDRL), Frederick, MD, from October 1980 to May 1981. The *U. scitaminea* teliospores were received and tested for germinability there, all inoculations were made there, and the inoculated plants were grown within the confines of the containment facilities. All materials that left the facility were sterilized either by pressurized steam or ethylene oxide to prevent the escape of smut spores to the surrounding area or to the Beltsville Sugarcane Quarantine facility about 45 miles to the southeast.

Sugarcane clones. Seven commercial sugarcane clones were used as test hosts: F 134, H 50-7209, H 68-1158, and NCo 310 were chosen because they had previously been used as differential hosts (3,7,10,12); CP 63-588, CP 65-357, and CP 70-1133 were selected because of their importance to the U.S. sugarcane industry. Nine months before the experiment began, cuttings of all clones except F 134 were treated with hot water (51 C) for 2 hr. The treated cane was propagated as single-bud cuttings in the quarantine greenhouse at Beltsville, MD, to produce the seed cane necessary for the experiment at PDRL. Clone F 134 was shipped from Canal Point, FL, to Beltsville the week of the experiment; the 8-mo-old cane had been field-grown and treated with hot water for 45 min at 52 C to eradicate *U. scitaminea* contamination.

Isolates of *U. scitaminea*. Smut isolates were collected by Victor Hemsy in Tucuman, Argentina (Ar), J. L. Dean in Florida (F), J. C. Comstock in Hawaii (A and B), W. H. Hsieh in Tainan, Taiwan (T_1 and T_2), and K. E. Cackett in Chiredzi, Zimbabwe (Z). Smut whips were collected in the field and shaken vigorously to release the teliospores. These were dried over a desiccant, packed in airtight vials, and sent to PDRL. Upon receipt at PDRL and again just before inoculation, the spores were tested for viability on 1% sucrose agar. The germination percentage of teliospores was determined from plates held 6 hr at 21 C after inoculation. Spores were stored over a desiccant at 21 C until used for inoculations of cuttings.

Inoculation and planting procedure. Inoculations were done by a hypodermic

Accepted for publication 27 August 1982.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

Table 1. Percentage of sugarcane clones infected with *Ustilago scitaminea* after hypodermic inoculation[a]

Isolate[b]	Clone						
	CP 63-588	CP 65-357	CP 70-1133	F 134	H 50-7209	H 68-1158	NCo 310
Ar	1/12[c](8)[d]	6/17 (35)	18/2 (69)	1/4 (25)	15/23 (65)	16/25 (64)	9/25 (36)
F	0/14 (0)	10/27 (37)	19/26 (73)	1/9 (11)	34/36 (94)	6/25 (24)	6/23 (26)
A	0/13 (0)	20/29 (69)	20/32 (63)	0/9 (0)	2/26 (8)	10/26 (38)	0/30 (0)
B	1/18 (6)	9/18 (50)	8/17 (47)	3/12 (25)	26/32 (81)	8/28 (29)	0/25 (0)
T_1	0/21 (0)	8/17 (47)	18/27 (67)	7/12 (58)	15/31 (48)	8/32 (25)	6/33 (18)
Z	1/13 (8)	17/22 (77)	16/33 (48)	8/13 (62)	27/32 (84)	10/25 (40)	19/33 (58)

[a] Controls of each clone were all negative and were not included in the table.
[b] Ar = Argentina, F = Florida, A = Hawaii A, B = Hawaii B, T_1 = Taiwan 1, and Z = Zimbabwe.
[c] Plants infected/plants living.
[d] Percentage of infected plants.

Table 2. Probability levels associated with significant differentiation of isolates among all possible pairwise comparisons among six isolates of *Ustilago scitaminea* on five sugarcane clones[a]

Isolate pairs	Clone				
	CP 65-357	F 134	H 50-7209	H 68-1158	NCo 310
Ar:F	...	...	5.4×10^{-3}	4.8×10^{-3}	...
Ar:A	2.8×10^{-2} [b]	...	2.6×10^{-5}	...	3.2×10^{-4}
Ar:B	...	...	...	1.0×10^{-2}	8.2×10^{-4}
Ar:T_1	...	...	...	3.4×10^{-3}	...
Ar:Z	9.9×10^{-3}	...	...	...	...
F:A	1.6×10^{-2} [b]	...	9.8×10^{-13}	...	4.4×10^{-3}
F:B	...	...	...	...	8.2×10^{-3}
F:T_1	...	3.8×10^{-2} [b]	2.1×10^{-5}	...	...
F:Z	5.2×10^{-3}	2.5×10^{-2} [b]	...	...	1.9×10^{-2} [b]
A:B	...	...	1.0×10^{-8}	...	...
A:T_1	...	6.8×10^{-3}	7.6×10^{-4}	...	1.6×10^{-2} [b]
A:Z	...	4.0×10^{-3}	2.2×10^{-9}	...	1.3×10^{-7}
B:T_1	...	...	6.3×10^{-3}	...	2.7×10^{-2} [b]
B:Z	...	...	...	...	8.6×10^{-7}
T_1:Z	...	...	2.6×10^{-3}	...	1.0×10^{-3}

[a] Probabilities calculated by Fisher's exact test for 2×2 tables.
[b] Significance between the 0.05 and 0.01 levels. All others significant at or below the 0.01 level.

injection technique (15) that was refined by Ferreira and Comstock (4). Sugarcane stalks were stripped of all leaves, cut into three-bud setts, then given a hot water treatment for 10 min at 52 C to stimulate growth. The setts, further cut into single-bud pieces, were dipped in a 300 mg a.i./L benomyl solution and put in wooden trays that had been thoroughly scrubbed with a 0.5% NaOCl solution and lined with absorbent paper saturated with the NaOCl solution. Sufficient 0.25% NaOCl solution was added to the trays (to a height of 1–2 mm) to eliminate saprophytic fungi and souring of sugar exudates by bacteria and yeast. The trays were sealed with plastic wrap, then placed in a greenhouse maintained at 30 C. Seed pieces germinated in 8–10 days. NaOCl (0.25%) was added as needed to keep the paper moist.

Shoots were inoculated when they were 8–12 cm long. The teliospore suspensions contained about 5×10^4 viable spores per milliliter of distilled water. To reduce surface tension, Tween 20 was added at a rate of 1 drop per 100 ml. The spore suspension was injected twice into each cutting at the base of the shoot (0.25 ml per injection) around the meristematic region, or until the inoculum was forced out the shoot tip. A 3-ml disposable syringe with a 1.25-cm, 26-gauge needle was used. After inoculation, the seed pieces were returned to the trays and allowed to incubate 1–2 days at 30 C. Most of the cuttings had a well-developed root system at the time of planting.

After incubation, two seed pieces were planted in each 15-cm clay pot filled halfway with a steam-pasteurized mixture consisting of equal parts sand, soil, and peat. The pot was then filled with more of the mixture so that part of the shoot tip was left above the soil line. The pots were arranged in a randomized block design with 12 seed pieces (six pots) used for each clone/isolate combination in each of four replicates. Uninoculated control plants of each clone were placed at random throughout the test. The greenhouse was maintained at 30 C and about 50% relative humidity with no added light.

Recording of disease incidence. After the cane was planted in October, the experiment was monitored closely for the appearance of the first smut whip. Thereafter, infected plants with smut whips were recorded, cut, and removed from the greenhouse at 10- to 14-day intervals until the experiment was terminated in late May. In April, it was necessary to move the remaining plants into an adjoining greenhouse section. Because of reduced bench area, one replicate had to be discarded. All plants discarded at that time and at the end of the experiment that had not shown the typical whip symptom were cut through the apical region to detect any juvenile whips that had not emerged from the spindle. Data were recorded and computed as the percentage of infection, with infection of a plant measured as the appearance of a smut whip (6).

RESULTS AND DISCUSSION

Infection data for the smut isolates on the seven clones of sugarcane are reported as the number of plants infected over the number of living plants, as well as infection percentages, because of the great variability in numbers of living plants (Table 1). The T_2 isolate is not included in the table because it infected only two plants in the test. Plants of F 134 germinated and developed poorly, reducing the number of plants of this clone in the final data.

Analysis of variance of the percentages of infected plants transformed to the arc sines showed significance ($P \leq 0.01$) for the interaction of clones with isolates (Table 2). This indicates differential interactions of the kind described by Vanderplank (18) as indicating vertical resistance in the host and races of the pathogen.

Because the data are based on small whole numbers, it is doubtful that they conform to the parametric assumptions. Therefore, further analysis to identify specific race differences was based on Fisher's exact test for 2×2 contingency tables, a nonparametric test with no restrictions on the values in any cell of the table (13). The 15 possible pairwise comparisons among six isolates were tested for significance on each of the seven clones. No pairs were found to be significantly different on CP 63-588 or CP 70-1133. Isolate pairs were separated on the remaining five clones (Table 2). Where a separation is shown, the probability that the separation is false (as calculated by the Fisher exact probability test) is also shown.

Members of each possible pairing of isolates were separated from each other on at least one differential clone. H 50-7209 and NCo 310 were outstanding with respect to both the number of pairs

separated and the low probability of error associated with most of those separations. Apparently these two clones are unusually useful as differential hosts.

Several of the clones used in the experiment were chosen because of their known differential reaction under field conditions at the locations from which the isolates came. H 50-7209 reacted as expected (5) with isolates A and B in the greenhouse test, and NCo 310 reacted as expected to the Z (8) and Ar isolates (N. E. Vazquez de Ramallo, *unpublished*). Clones F 134 and H 50-7209, however, appeared to be susceptible to the T_1 isolate in the greenhouse but were reported resistant in Taiwan (12). In addition, the greenhouse data with isolates A and F on NCo 310 contradict previous field results in Hawaii (5) and Florida (6). Results obtained with the dip-inoculation method in the field are variable from test to test and may give a false reading. Several tests are necessary for reliable smut/clone reaction data. The discrepancies between our data and those in the literature may involve differences in inoculation methods or environment or they may even involve a difficulty in maintaining a pure genetic line of the pathogen. The greenhouse method described here is not to be considered as a substitute for screening for smut resistance under field conditions prevalent at a particular location, but it is a method for evaluating smut/isolate differences under uniform conditions.

Tests were performed between October 1979 and March 1980 in the same greenhouse with five of the same clones (A. G. Gillaspie and R. G. Mock, *unpublished*). The dip method of inoculation (2) was used for the B and F isolates. When the test results were compared with data presented here, there were no significant ($P \leq 0.01$) differences between the results obtained for any of the isolate-clone combinations except for NCo 310 and Hawaii B isolate. This increases confidence in the repeatability of these results.

Clones varied in the time required for appearance of smut whips. The F isolate produced whips on 55% of the H 50-7209 plants 5 mo after inoculation, but on NCo 310, there were no whips after 6 mo, and whips were present in 26% of the plants after 7 mo. Another example of differing smut susceptibilities involved the Ar isolate. Percentages of infection of Ar on H 68-1158 and H 50-7209 were almost identical 7 mo after inoculation (64 and 65%, respectively). At 5 mo postinoculation with Ar, however, H 68-1158 had only 12% infection and H 50-7209 had 52% infection. Clone H 50-7209 differed from the other six in its overall response (all isolates pooled) by producing a peak number of new whips at 5 mo after inoculation and then a decreasing number thereafter. The other clones showed an increasing number of new whips over the entire period.

When the data were combined by isolate for reactions on all clones, the Z isolate was found to produce 60% infection. The other isolates (excluding T_2) ranged from 50% down to 30% infection in order as follows: Ar, F, B, A, T_1. Isolate Z peaked at 5 mo in new whips produced, but the other isolates increased gradually over the 7 mo.

Because the teliospores of the isolates were collected in widely separated areas of the world by different workers at different times, it is apparent that environmental effects on the spores may be confounded with genetic differences among isolates in this test. The method used in the pairwise comparison of isolates on each clone would not distinguish between genetic and environmental effects. However, if important environmental effects were involved, they would have to be clone-specific to lead to an interaction of clones with isolates, and this seems unlikely. The most likely environmental effect on the spore inoculum would be a nonspecific loss of germinability or of pathogenicity. Adjustment of inoculum concentration compensated for differences in germinability. Even though a loss of pathogenicity would be confounded in the results, this loss is not likely to be clone-specific.

The greenhouse environment is very different from those of the areas where the spores were collected but it allowed comparison of isolates and clones under the same conditions and techniques. Each of the six isolates probably represents a different race of *U. scitaminea* with important differences in clonal host range. This indicates that races of the fungus will probably complicate breeding for smut resistance. This greenhouse method is a valid, rapid method for isolate separation when the correct differential clones are used.

ACKNOWLEDGMENTS

The technical assistance of Dennis Johnson, Walter Sistrunk, Samuel Little, and Russell Harris is gratefully acknowledged.

LITERATURE CITED

1. Antoine, R. 1961. Smut. Pages 326-354 in: Sugar-cane Diseases of the World. Vol. 1. J. P. Martin, E. V. Abbott and C. G. Hughes, eds. Elsevier Publishing Co., Amsterdam. 542 pp.
2. Byther, R. S., and Steiner, G. W. 1974. Comparison of inoculation techniques for smut disease testing in Hawaii. Proc. Int. Soc. Sugar Cane Technol. 15:280-287.
3. Comstock, J. C., and Heinz, D. J. 1977. A new race of culmicolous smut of sugarcane. Sugarcane Pathol. Newsl. 19:24-25.
4. Ferreira, S. A., and Comstock, J. C. 1981. A new inoculation technique for sugarcane smut, *Ustilago scitaminea*. (Abstr.) Phytopathology 71:873.
5. Ferreira, S. A., Comstock, J. C., and Wu, K. K. 1980. Evaluating sugarcane smut resistance. Proc. Int. Soc. Sugar Cane Technol. 17:1463-1476.
6. Holder, D. G., and Dean, J. L. 1979. Screening for sugarcane smut resistance in Florida: Second preliminary report. Sugar J. 41(11):18-19.
7. James, G. L. 1969. Smut susceptibility testing of sugarcane varieties in Rhodesia. Proc. S. Afr. Sugarcane Technol. Assoc. Annu. Congr. 43:85-92.
8. James, G. L. 1973. The effect of smut infection on sugar yield. Sugarcane Pathol. Newsl. 10:32-33.
9. Koike, H., Fontenot, D., Damann, K., and Schlub, R. 1981. Smut of sugarcane in Louisiana: A first report. Plant Dis. 65:1018.
10. Ladd, S. L., and Heinz, D. J. 1976. Smut reaction of non-Hawaiian sugarcane clones. Sugarcane Pathol. Newsl. 17:6-14.
11. Lee-Lovick, G. 1978. Smut of sugarcane—*Ustilago scitaminea*. Rev. Plant Pathol. 57:181-188.
12. Leu, L. S., and Teng, W. S. 1972. Pathogenic strains of *Ustilago scitaminea* Syd. Sugarcane Pathol. Newsl. 8:12-13.
13. McGuire, J. V., Lehmann, R. P., and Heath, A. L. 1967. Pages II and III in: Tables of Exact Probabilities for 2 × 2 Contingency Tests. ARS Handbook 20-15. 243 pp.
14. McMartin, A. 1945. Sugarcane smut: Reappearance in Natal. S. Afr. Sugar J. 29:55-57.
15. McMartin, A. 1948. Sugarcane smut. S. Afr. Sugar J. 32:749.
16. Raja, K. T. S., Natarajan, S., Ahmed, N. J., and Padmanabham, D. 1972. Effect of smut incidence in plant and ratoon crops on yield and quality. Sugarcane Pathol. Newsl. 9:9-10.
17. Todd, E. H. 1978. Sugarcane smut in Florida. Sugar J. 41(3):23.
18. Vanderplank, J. E. 1975. Principles of Plant Infection. Academic Press. New York. 216 pp.
19. Villalon, B. 1982. Sugarcane smut (*Ustilago scitaminea* Syd.) in the lower Rio Grande Valley of South Texas. Plant Dis. 66:65-66.
20. Whittle, A. M. 1978. Thoughts on smut resistance testing. Sugarcane Pathol. Newsl. 20:43-46.