

THE DEVELOPMENT OF AN IMPROVED METHOD FOR EVALUATING SUGARCANE FOR RESISTANCE TO SMUT

By H. L. LLOYD and M. PILLAY

Department of Microbiology, University of Durban-Westville

Abstract

The isolation from bud scales and partial characterisation of highly active, low molecular weight, germination inhibitors, in concentrations correlated quantitatively with resistance of N52/219, N11, NCo376, and NCo293, holds promise for the development of a rapid, quantitative, chemical assay for evaluating sugarcane for pre-infectious resistance to smut. Histopathological studies on stalk and meristem colonization by *Ustilago scitaminea* did show, however, that there are post-infectious forms of resistance that may be independent of pre-infection resistance. The two forms of resistance may be the basis of differential varietal response to smut races.

Introduction

The generally used system for the evaluation of varieties for smut resistance, in which setts are dipped into teleutospore suspensions, planted out and numbers of whips counted in plant or ratoon crops after 6-12 months or more, was developed empirically¹⁵. The system has remained basically unchanged for over 20 years⁸ although its reliability has been frequently questioned^{4, 9, 10, 20} and the protracted period necessary for evaluations to be made is a limitation to an ongoing breeding programme.

Alternative methods tested which seem not to improve the efficiency or speed of evaluation have been directed at improving and standardizing the application of spores to the nodal bud^{4, 6, 12, 13, 17, 19, 22}. However, James¹⁰ concluded that all methods which included artificial inoculation of the bud gave anomalous evaluations of resistance and suggested that natural infection was more dependable. Natural methods invariably have a large "disease escape" factor with which to contend. Furthermore the correlation ($r = 0,70$) of results between natural infection and the traditional immersion inoculation testing obtained by Hawaiian workers⁸ over many years is no better than the correlation ($r = 0,728$) between the inhibition of spore germination on internode surfaces and the resistance of forty varieties¹¹ or the correlation ($r = 0,708$) between resistance and bud morphology²¹. A radically different approach is thus indicated if there is to be developed a rapid, more reliable smut resistance evaluation system applicable to early phases of a sugarcane breeding programme.

The nodal bud was originally suspected to be the site of primary infection^{1, 7, 15} and this was later established experimentally^{2, 4, 21}. Non-germinated buds may become infected in the soil after planting setts^{4, 14}, or germinating spores, previously channelled down leaf sheaths, may infect buds on standing cane^{5, 13, 20}.

The observation that the germination of spores on the bud is inhibited in proportion to the resistance status of the variety¹¹, and that spore injections beneath the bud scale¹⁵ and the removal of outer bud scales prior to inoculation²⁰ increased the percentage infection all strongly suggest that the outer bud scales protect, either chemically¹¹ or physically^{7, 16}, the bud from infection. Lower buds seem to be more effectively protected than the upper buds on the same

stalk⁴ and inoculation of shoots becomes increasingly ineffective with ageing, i.e., length⁴. This suggests that scale leaves on developing shoots may also protect sugarcane from smut infection.

The penetration of bud scales of susceptible varieties by promycelial branches is preceded by appressorium formation²¹. Subsequent etiological events have not been detailed. Mycelial establishment and colonization during the prolonged period between the penetration and whip formation phases of the infection cycle are vague and described only recently for a very susceptible variety Co213². Information does not extend to the differences in colonization of resistant and susceptible varieties. However, a larger number of stalks form whips and whips develop earlier in more susceptible varieties^{10, 16, 20} indicating that the rates and patterns of colonization differ in susceptible and resistant tissues. This, in turn, suggests that there are internal resistance factors, even though evidence points to some property of the bud (and leaf) scale as a primary barrier to infection.

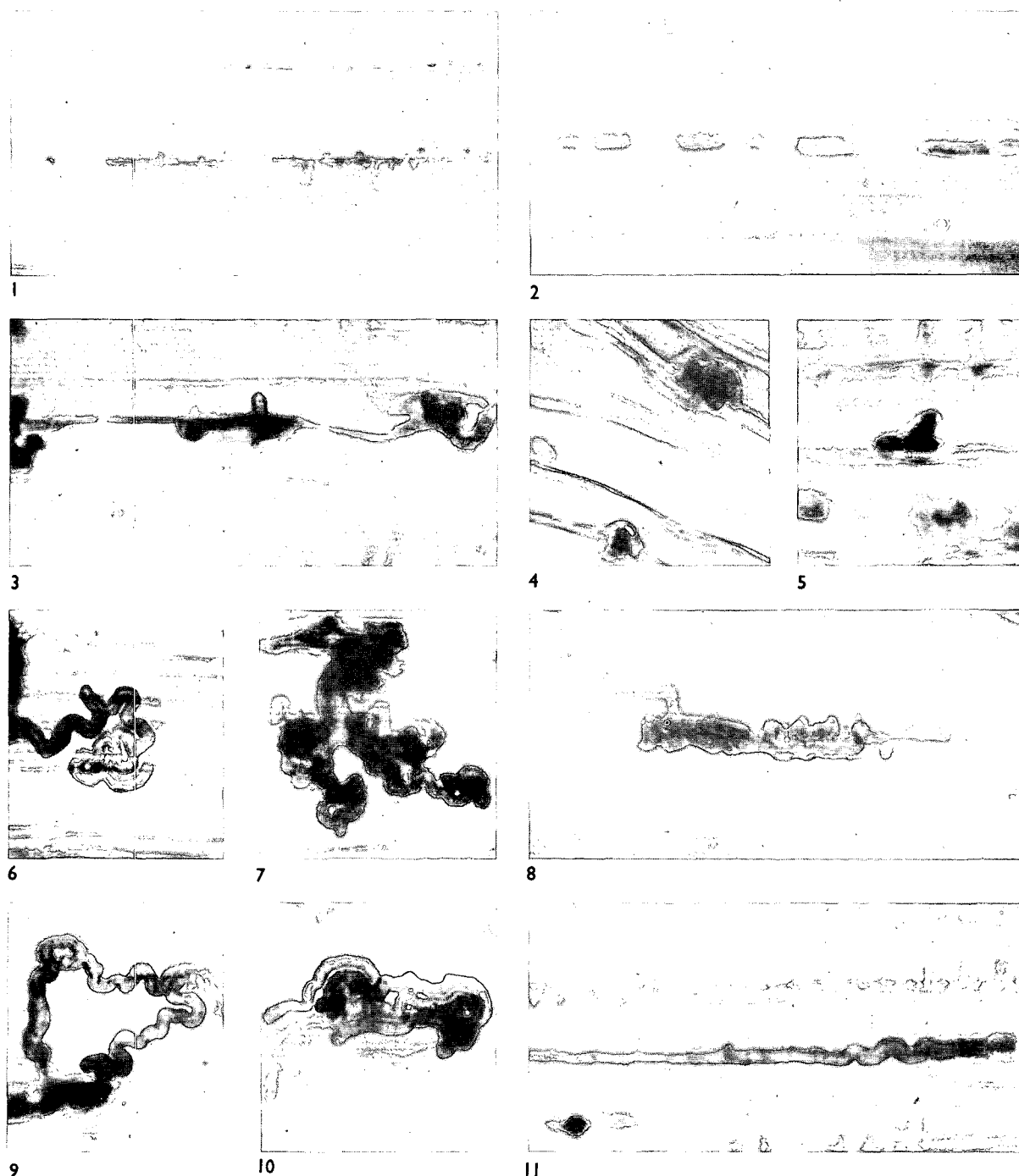
Any different approach to evaluating sugarcane for smut resistance will need to be based on better and more complete knowledge about the etiology of smut, the nature of resistance and at what stage in the etiological cycle of smut resistance is manifested. This paper describes previously unknown histopathological events during the stalk colonization phase of the etiological cycle of smut which, together with other etiological details^{2, 21}, was necessary information for the formulation of a working hypothesis on the location and nature of resistance, which in turn served as a basis for the preliminary development work, reported here, on a chemical assay for the evaluation of smut resistance in sugarcane.

Methods and Materials

A quantitative comparison of smut infection and inhibitors present in bud scales was carried out on four varieties: N52/219, N11, NCo376 and NCo293, with smut resistance ratings on the international scale⁸ of 1, 3, 7 and 9 respectively³.

Eight centimetre long, single-budded setts were cut from the mid-third region of nine month-old canes, washed for 30 seconds in 0,25% calcium hypochlorite and pregerminated for 6-8 days in the dark at 25°C. Uniformly germinated setts were potted in fumigated soil. Greenhouse conditions were controlled to provide a regular 12 h alternating cycle of 32°C during the day and 20°C at night and a midday RH of 60%. The potted setts were arranged in a randomised blocks design with four blocks (= varieties), each with three plots of 20 potted plants. One plot served as the uninoculated control, one plot was inoculated four days after pruning back shoots to stimulate tillering and in the third plot, buds of pregerminated setts were inoculated at the time of potting. A 0,2 ml suspension of teleutospores (5×10^4 spores/ml) previously determined to have a germination percentage of 32% at 25°C was applied to each bud.

From one to eight months after inoculation samples of main stem and/or tillers from plants representing each inoculation treatment were removed and cut into two centimetre long, serially numbered sections. Each cylindrical stalk



FIGURES 1-11 intercellular hyphae, the distribution and development of haustoria and haustorial morphology of *Ustilago scitaminea* in stalk and meristem tissues of resistant and susceptible sugarcane varieties.

section was halved and then quartered lengthwise. One 0,5 cm x 2 cm x 1 mm tissue section (including the rind in each section) was cut from each of the two tissue faces at right angles to each other on the quartered stem section. Tissue sections were cleared in lactophenol (BDH Chemicals, Ltd.) and stained with a solution of 1,5% cotton blue in lactophenol (w/v). Colonization was quantified using a 10x objective and a squared eyepiece graticule (1 mm squares) by counting the total number of squares occupied by haustoria in 10 microscope fields at equal distances along the 2 cm section. Two scans 3 mm apart of 10 fields each along the length of the section were made for each tissue section and the mean number of squares occupied by haustoria per section calculated as the colonization index.

Material for chemical analysis and bioassays was obtained from 7 month ratoon cane formed over the period September — March 1978/79. Single-eyed setts were cut from

stalks and bud scales were stripped from buds within 24 h of harvest and processed immediately.

Half-gram samples of bud scales from the lower-third section of the stalk of varieties N52/219, N11, NCo376 and NCo293 were homogenised in 50 ml acetone/petroleum ether (9 : 1 v/v), filtered and evaporated to dryness *in vacuo* at 30°C. The dried film was extracted sequentially with petroleum ether (BP 60-80°C), chloroform, diethyl ether, acetone and methanol. Each extract was dried *in vacuo* and re-dissolved in 0,5 ml of the same solvent. The extracts were subjected to HPLC analysis using a methanol/water (90 : 10 v/v) solvent and micro bondpak C18 column (Waters Associates). Components of each extract, separated by TLC analysis on cellulose plates (Merck) using butanol/acetic acid/water (4 : 1 : 5), were located in shortwave uv light, extracted quantitatively with water and tested for spore germination inhibitory properties.

Inhibition of germination at 25°C and 30°C in the dark was assayed by mixing equal volumes of the aqueous extract of the TLC separated component and a 3×10^3 /ml suspension of teleutospores. Equivalent areas adjacent to the separated compounds on TLC plates were extracted with water and bioassayed as controls.

Single-budded setts of varieties N52/219, NCo376 and NCo293 were surface sterilized in 0,2% calcium hypochlorite, inverted with the bud area held in 0,1 ml aqueous suspensions of teleutospores or distilled water (as controls) for 18 h at 25°C in the dark. After incubation the droplets were collected, bulked (2-3 ml), centrifuged at 2 400 rpm for 10 min and the supernatant was extracted with 2×5 ml diethyl ether. Extracts were evaporated to dryness and redissolved in 0,2 ml methanol for HPLC and TLC analysis.

Results

Colonization of resistant and susceptible stalk tissue by *U. scitaminea*.

The rate of colonization by *Ustilago scitaminea* of main shoots, tillers and roots was correlated ($r = 0,89$) with the resistance rating of varieties N52/219, N11, NCo376 and NCo293 (Table 1). The extent of hyphal colonization, based on the colonization index, which is a numerical index of the number, size and distribution of haustoria, was similar in all varieties.

The colonization hyphae and haustoria were not uniformly distributed laterally in the stalk and in all varieties they were associated with periferal vascular bundles. Haustoria formed predominantly in protoxylem, lysigenous cavities

TABLE 1

The mean rate and degree of colonization by *Ustilago scitaminea* of sugarcane stalk tissue from four varieties differing in resistance to culmicolous smut.

Varieties	N52/219	N11	NCo 376	NCo 293
Resistance rating . .	1	3	7	9
Colonization index .	13,46	11,33	14,50	12,50
Colonization rate (mm/day)	1,12	1,07	1,26	1,54

and tracheids in NCo376, N11 and N52/219 and in xylemparenchyma of the vascular bundle in NCo293. The vertical distribution of *Ustilago scitaminea* from the base of the stalk (crown) to the sporulation zone in N52/219, N11, NCo376 and NCo293 was a repetitive pattern of higher concentrations, and a greater lateral distribution, of haustoria in nodal-compared to internodal areas. Heavier colonization of nodal regions and the preponderance of hyphae in periferal vascular bundles seems to provide a ready access to nodal buds and thus to tillers and roots arising from the crown region.

The colonizing intercellular hyphae are restricted to sclerenchymatous tissue and between tracheids of the vascular bundle (Figure 1). Haustorial mother cells (HMC) developed initially as swellings at regular intervals along the intercellular hyphae (Figure 2). HMC thickened and elongated and a short lateral branch of similar diameter (the haustorial neck) protruded from HMC at right angles to the wall of the adjacent tracheid or xylemparenchyma cell (Figure 3).

The protruding haustorial neck and mother cell in the more resistant varieties N11 and NCo376 were initially enveloped

by a thickened and invaginated tracheid wall (Figure 4) or sclerenchyma cell wall (Figure 5). In NCo293 the developing haustorial neck readily traversed the cell wall and did not seem to be surrounded by host cell wall material (Figure 3).

Haustoria formed in lysigenous cavities, protoxylem, tracheids or xylemparenchyma cells adjacent to intercellular hyphae between tracheids and in sclerenchymatous tissue and were spiralled and either unilobed (Figure 6) or multi-lobed (Figure 7). Haustorial form differed in resistant and susceptible tissues, however. In varieties NCo376, N11 and N52/219 haustoria were tightly coiled with multiple, short lobes (Figure 8), while colonized tissues of NCo293 had fewer, long, loosely coiled lobes when formed in xylemparenchyma cells (Figures 9, 10) or single, very long, spiralled lobes in tracheids (Figure 11).

Quantification of spore germination inhibitors in resistant and susceptible varieties

The acetone extract only of the five sequential solvent extractions analysed by HPLC gave an inverse quantitative relationship between peak height and varietal resistance to smut (Figure 12). The close inverse relationship between peak area (= concentration) and the corresponding varietal resistance ratings is shown in Table 2. Only the acetone extract was included in subsequent analyses and bioassays.

TABLE 2

The mean concentration (=peak area) of the sum of compounds in the acetone extracts from varieties N52/219, N11, NCo 376 and NCo 293 resolved as a single peak by HPLC analysis.

Variety	N 52/219	N11	NCo 376	NCo 293
Resistance rating . .	1	3	7	9
Concentration (peak area cm ²) . .	4,15	3,82	2,33	2,05

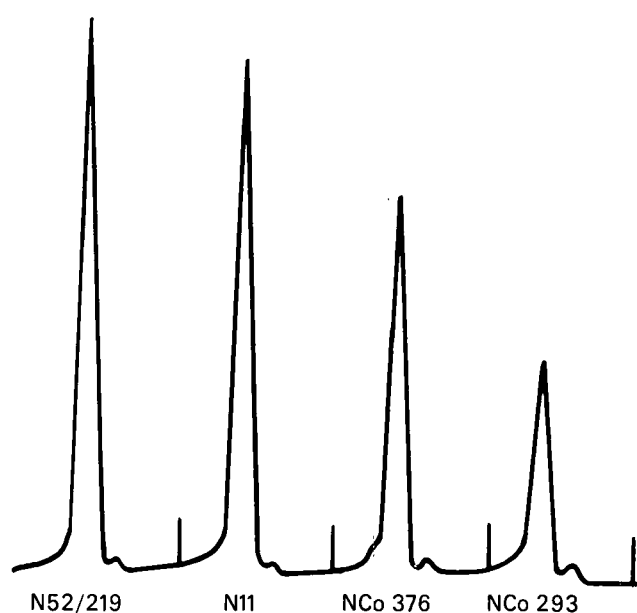


FIGURE 12 The concentration (=peak area) of the sum of flavonoid germination inhibitors and related minor compounds resolved as a single major peak by HPLC analysis of extracts from bud scales of N52/219, N11, NCo 376 and NCo 293.

TLC analyses of acetone extracts resolved four major structurally related flavonoid glycosides with characteristic absorption maxima at 272 nm and 202 nm. These major components together with several related minor compounds

in the acetone extract were resolved as a single major peak under the operational parameters of HPLC analysis. The relative concentration of these major components differed between varieties and their relative proportions were not consistent with the resistance of varieties. Each of the major components in the acetone extracts from N52/219 inhibited teleutospore germination at 25°C but caused no significant inhibition of germination at 30°C (Table 3).

TABLE 3

The mean percentage germination of teleutospores in the dark at 25C and 30C in three concentrations of the four major flavonoid compounds resolved by TLC from the acetone extract of the variety N52/219.

Temperature . . .	25C				30C			
	F1	F2	F3	F4	F1	F2	F3	F4
Control	31,5	26,5	31,0	27,0	17,0	15,0	13,0	10,0
Conc. 100%	23,0	21,0	15,5	19,0	13,5	9,0	12,0	13,0
50%	18,5	10,5	14,5	13,0	13,0	8,5	9,5	9,5
25%	21,0	17,5	21,5	20,0	13,5	10,0	8,0	10,5

The diffusates from intact buds held in spore suspensions for 18 hours contained *inter alia* the same four major flavonoid glycosides. These compounds were again resolved as a single major peak and were at higher concentrations in buds held in spore suspensions than buds held in distilled water (Figure 13). The relative concentrations in the three varieties (N52/219, NCo376, NCo293) remained the same, however.

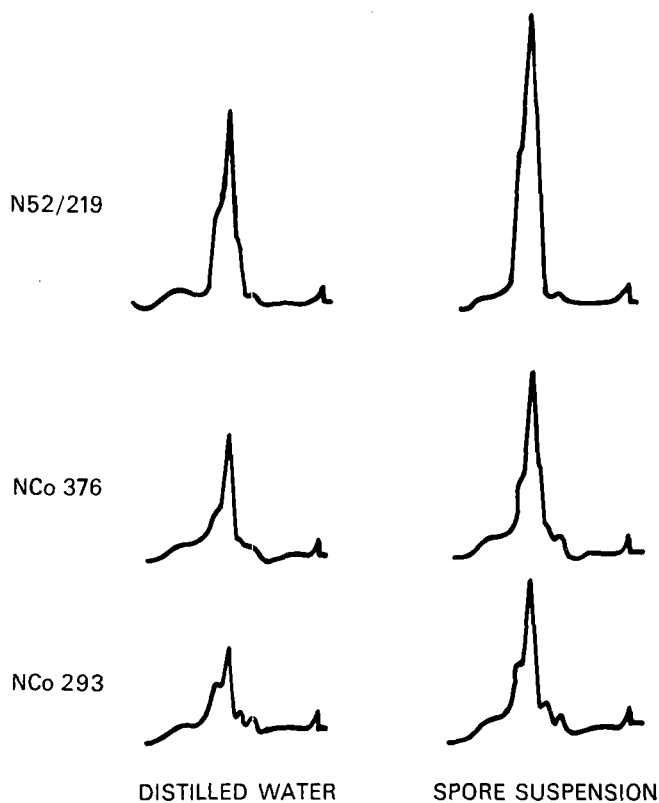


FIGURE 13 HPLC analysis of the the ether extractable diffusates from intact buds held for 18 h in distilled water (control) and spore suspensions of *Ustilago scitaminea*. The major peak in each case contained the compounds inhibitory to spore germination.

Discussion

Histopathological features of smut during the colonization phase of the etiological cycle suggests that, in addition to pre-infectious resistance associated with the bud scale, post-infectious barriers to colonization are present in the stalk and meristem. Preferential colonization of vascular tissue suggests that, in general, ground tissue is relatively inhospitable. The restriction of haustoria to structural tissues in the vascular bundle and the smaller size and morphology of haustoria in moderately and highly resistant varieties compared to large haustorial size and morphology and their presence in xylem-parenchymatous tissue in NCo293 indicates that there may be specific inhibitory substances at higher concentrations in the stalk tissue of resistant varieties. The proportional reduction in the colonization rate with increasing resistance is likely to be a function of reduced haustorial efficiency and/or rate of haustorial development. The latter is due either to mechanical cell wall barriers observed enveloping haustorial initials or to a direct toxin effect which reduces the rate and vigour of haustorial formation, thus providing time for the 'walling in' of haustorial initials in more resistant tissues.

The isolation and partial characterization of preformed teleutospore germination inhibitors from bud scales in concentrations correlated with varietal resistance corroborates the indirect observations^{4, 11, 21} that the bud has a protective function against *U. scitaminea*. Pre-infectious resistance associated with the bud scale is both static and dynamic. That is, the lower concentrations of inhibitory substances naturally present in bud scales are supplemented by *de novo* synthesis or transformation to active inhibitory forms in the presence of germinating smut spores on the surface of the bud scale.

Pre-infectious resistance reduces the probability of infection providing the bud scale is intact and the teleutospores cannot circumvent this barrier via bud grooves or injuries. Waller's²¹ data provide evidence for a correlation between bud morphology (looseness of bud scales and the presence of a bud groove) and susceptibility of sugarcane varieties to smut. By reducing the probability of infection, pre-infectious resistance will reduce the percent infection of stools. Most countries, not including Rhodesia^{8, 10} and South Africa (R. A. Bailey, pers. comm.), assign resistance ratings to sugarcane varieties using percent infection of stools. Post-infectious resistance reduces colonization rate of the meristem and stalk. This form of resistance will, after a given time interval, be manifested as a reduction in the number of smut whips formed per unit area. The latter method of evaluating varieties was recommended by James¹⁰.

Pre-infectious resistance is effected by reducing the germinable spore concentrations to levels below the minimum threshold of infection. This form of resistance is thus only effective when inoculum levels are sufficiently low for the inhibitors to cope. This is in accordance with the common practical recommendation for smut control that the planting of resistant varieties must be accompanied by the practice of roguing infected plants¹⁸.

The reduced sensitivity of germinating spores to inhibitors at 30°C compared to 25°C may partially explain why sugarcane smut is more severe in warmer regions.

The increased incidence of smut in NCo376 plant cane following hot water treatment¹⁸ may be due to the ready extractability of glycosidic inhibitors by a hot, polar solvent like water. The reduction in the concentration of inhibitors will increase the probability of infection of sett buds. Varieties with moderate levels of pre-infectious resistance con-

ditioned by moderate concentrations of inhibitors would be particularly prone to this hot water treatment effect.

The isolation from bud scales of preformed, readily identifiable, low molecular weight inhibitor compounds in concentrations commensurate with the resistance status of varieties is a promising development for establishing a rapid, chemical assay for evaluating sugarcane for resistance to smut. The nature of post-infectious resistance and its status *vis a vis* pre-infectious resistance and the independence of these two forms of resistance must be established before a chemical assay procedure, which will determine levels of pre-infectious resistance only, can be implemented. James¹¹ data on two out of 40 varieties gives some indication that post-infectious resistance may be important and independent of pre-infectious resistance. That is, spore germination was not inhibited on the buds of varieties M31-45 and Co1001, both of which have smut resistance ratings of 1.

The existence of two, possibly independent, forms of resistance and at least in one of them, several participating compounds, provides the potential permutations to account for the observed differential varietal reaction to races A and B of *U. scitaminea*. Of possible significance to the independence of pre- and post-infectious resistance is the finding that resistance to smut race A and B is inherited independently⁸.

Acknowledgements

The work reported in this paper forms part of a research programme financed at the University of Durban-Westville by the South African Sugar Association. Both the financial assistance from SASA and the co-operation received from the Director and Staff, particularly Dr. J. Allison, R. A. Bailey and K. J. Nuss, of the S. African Sugar Association Experiment Station, is gratefully acknowledged.

REFERENCES

1. Ajrekar, S. L. (1916). On the mode of infection and prevention of the smut disease of sugarcane. *Indian Agric. J.* **11**: 288-295.
2. Alexander, K. C. and K. Ramakrishnan (1980). Infection of the bud, establishment in the host and production of whips in sugarcane smut (*Ustilago scitaminea* Syd.) of sugarcane. *ISST Proc XVII* (in press).
3. Bailey, R. A. (1979). An assessment of the status of sugarcane diseases in South Africa. *SASTA Proc* **53**: 120-128.
4. Bock, K. R. (1964). Studies on sugarcane smut (*Ustilago scitaminea*) in Kenya. *Trans. Brit. mycol. Soc.* **47**: 403-417.
5. Chona, B. L. (1943). Sugarcane smut and its control. *Indian Fmg.* **4**: 401-404.
6. Early, M. P. (1970). Current programmes in sugarcane disease research centres (6) Kenya. *Sug. Path. Newsletter* **5**: 32.
7. Fawcett, G. L. (1944). 'Carbon de la Caña de Azucar. Bol. Estac. exp. agric. Tukuman No. 147.
8. Ferreira, S. A.; J. C. Comstock and K. K. Wu (1980). Evaluating sugarcane smut resistance. *ISST Proc XVII* (in press).
9. James, G. L. (1969). Smut susceptibility testing of sugarcane in Rhodesia. *SASTA Proc* **43**: 85-92.
10. James, G. L. (1972). Smut incidence in variety trials. *SASTA Proc* **46**: 211-215.
11. James, G. L. (1973). Smut spore germination on sugarcane internode surfaces. *SASTA Proc* **47**: 1-2.
12. Leu, L. S. (1971). Reaction of clones of *Saccharum spontaneum* to *Ustilago scitaminea* Syd., the causal organism of the culm-colour smut of sugarcane. *Sug. Path. Newsletter* **7**: 10-11.
13. Luthra, J. C.; A. Sattar and S. S. Sandu (1938). Life history and modes of perpetuation of smut of sugarcane (*Ustilago scitaminea*). *Indian J. Agric. Sci.* **8**: 849-862.
14. McMartin, A. (1945). Sugarcane smut: reappearance in Natal. *S. Afric. Sugar J.* **29**: 55-57.
15. McMartin, A. (1948). Sugarcane smut. *S. Afr. Sugar J.* **22**: 737-749.
16. Robinson, R. A. (1959). Sugarcane smut. *East Afr. Agric. J.* **24**: 240-243.
17. Sandu, S. S. and N. S. Mann (1966). Varietal resistance to sugarcane smut caused by *Ustilago scitaminea* in the Punjab. *J. Res. Ludhiana* **3**: 410-413.
18. South Afr. Sug. Assoc. Expt. Sta. (1977). Sugarcane production in South Africa. Bull. No. 1 (revised), 44 pp.
19. Srinivasan, K. R. (1969). Methods for testing the resistance of sugarcane to disease 5. Sugarcane smut. *Sug. Path. Newsletter* **2**: 7.
20. Waller, J. M. (1970). Sugarcane smut (*Ustilago scitaminea*) in Kenya I. Epidemiology. *Trans. Brit. mycol. Soc.* **52**: 139-151.
21. Waller, J. M. (1970). Sugarcane smut (*Ustilago scitaminea*) in Kenya II. Infection and resistance. *Trans. Brit. mycol. Soc.* **54**: 405-414.