A NECROTROPHIC REACTION IN *NICOTIANA* SPECIES INDUCED BY *PERONOSPORA TABACINA* ADAM

II.* QUANTIFICATION OF THE RESISTANCE–SUSCEPTIBILITY STATUS OF TOBACCO BREEDING LINES

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Summary

A comparison was made between the performances of a large-plant test, the cotyledon test, and a test based on the necrotrophic reaction for assessment of the resistance–susceptibility status of various tobacco species and breeding lines to infection by *Peronospora tabacina* Adam. All three methods were shown to give similar assessments and it is suggested that the necrotrophy test gives an accurate measure of intrinsic resistance.

The necrotrophy test has an advantage that there is rigid control of environmental factors, and tests using fungal strains can be made on a single plant at a comparatively early stage of growth. As the necrotrophy test is performed on leaf disks, the plant is not infected and is available for future breeding work.

Measurements of mycelial growth rate showed that cv. Virginia Gold was a more congenial host for *P. tabacina* strain APT1 than any other of the hosts tested. In the case of mould strain APT2, highest mycelial growth rates were found in tobacco breeding lines resistant to mould strain APT1. Mould strain APT2 showed a lower growth rate in cv. Virginia Gold than mould strain APT1.

I. INTRODUCTION

The assessment of resistance–susceptibility status of tobacco lines to *Peronospora tabacina* Adam is conventionally made following the total infection of plants by the fungus. Two approaches are currently in use, one using plants with true leaves and the other involving infection of cotyledons.

In the first approach, seedlings or large plants are inoculated with conidial suspensions of the fungus, kept in an environment suitable for infection and mould development for 6–8 days, and then disease severity is rated. The various scales used for rating depend on assessment of numbers of infected leaves, severity of symptoms, and amount of sporulation present (Izard, Schiltz, and Hitier 1961; Hill and Mandryk 1962; Ternovskii and Dashkeyeva 1963).

In the second approach, the cotyledon test of Izard and Schiltz (1963), plants are classed as susceptible or resistant on the basis of whether or not sporulation occurs on inoculated cotyledons. This test is of particular use in assessing the degree of resistance of a plant population and in selection for subsequent resistance breeding.

Both tests involve the loss of susceptible plants examined. While the cotyledon test is performed under rigidly controlled environmental conditions, such control is

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rarely, if ever, achieved in practice in large-plant tests. Thus, in the latter case, the final assessment is dependent not only on the inherent resistance–susceptibility status of the plant, but also is influenced to a variable degree by environmental factors. Some attempt has been made to estimate the effects of these factors (Hill 1965; Hill and Green 1965) but, because of the large numbers of plants required for testing, this more elegant approach has not yet been practised in resistance–susceptibility assessments by plant breeders.

Shepherd and Mandryk (1967) observed that, under specific conditions, a necrotrophic reaction was produced in various *Nicotiana* species following infection with *P. tabacina* and reported that the reaction was most marked in those species susceptible to the organism. They also noted that hybrids of *N. tabacum* × *N. debneyi* gave a variety of reactions and that, in general, there appeared to be correlation between severity of reaction and the resistance rating of the hybrids.

The purpose of the present study was to examine in detail the relationships between the necrotrophy test and the resistance–susceptibility status of various tobacco breeding lines, as determined by both large-plant and cotyledon tests.

II. Materials and Methods

A range of eight breeding lines of varying resistance–susceptibility status to blue mould were kindly made available by Mr. D. C. Wark, Division of Plant Industry, CSIRO. Seeds were germinated in vermiculite and seedlings were transplanted into 6-in. pots filled with perlite. Fifteen plants of each breeding line, of *N. debneyi* Domin, and of *N. tabacum* cv. Virginia Gold were grown under shade (50% Sarlon cloth) at a day temperature of 22°C and a night temperature of 16°C for a 14-hr day in the CSIRO phytotron at Canberra (Morse and Evans 1962). Plants were given Hoagland's solution each morning and tap water each evening.

For the necrotrophy test, disks (10 mm diam.) were cut from the lower leaves of 6-week-old plants and floated, ventral surface uppermost, on an aqueous solution of 1 μg/ml kinetin (Shepherd, Stuart, and Mandryk 1963). Twenty disks were taken from each plant and inoculated with a standardized conidial suspension and incubated as described by Shepherd and Mandryk (1967). After incubation for 48 hr, the disks were examined individually under a stereomicroscope for the recording of symptoms, and then photographed as described by Shepherd and Mandryk (1967) before being returned to the incubation conditions. The disks were re-examined and photographed at 24-hr intervals for a further period of 8 days.

The intensity of the necrotrophic reaction was rated according to the following scale:

0, no reaction;

1, water soaking only, no necrosis;

2, a few isolated necrotic cells under the inoculation drop only;

3, many isolated necrotic cells or necrotic areas or both under the inoculation drop only;

4, necrotic cells occurring both inside and outside the inoculation drop area, but those outside occurring only at the periphery of the drop;

5, necrotic cells spread beyond the drop margin, but over less than half the diameter of the leaf disk;

6, necrotic cells spread over more than half the diameter of the leaf disk.

The mean necrotrophic intensity per disk was calculated from replicates of 20 disks from each of the 15 plants (300 disks in all) in each breeding line.

The area of total necrosis was estimated by superimposing a transparent graticule onto a projected image of the photographs obtained by the method referred to above. Because of the labour involved in such estimations, the area measurements for use in the formula below were calculated for a 96-hr incubation period only.

The results from the necrotrophic intensity rating tests and the area of total necrosis estimates were compounded to give a "combined necrosis" rating, using the formula:

$$\text{combined necrosis rating} = (\text{area} + \text{intensity}) \times \text{intensity},$$

where "area" is the relative area estimated after incubation for 96 hr. This combined rating gives a fuller description of the actual results observed, particularly in the case of necrotrophic reactions of intensities between 0 and 4, than either intensity or area used separately. The term (area + intensity) was used instead of area alone in order to include visible effects that did not involve necrosis, e.g. in the case of mould strain APT2 infection of the F_1 hybrid, water soaking was observed on all disks, while an occasional weak necrosis occurred on a small proportion of the disks. Furthermore, in some instances, disks from different species showed similar areas of total necrosis, but the intensity of the reaction, as judged by the actual number of necrotic lesions present, differed considerably (cf. lines SO1 and Bel-12 infected with mould strain APT1). The above formula is weighted in favour of intensity to cover such cases.

At intervals of 48 hr during the incubation period, two disks were taken from each sample and examined for the presence of mycelium by the method of Isaac (1960). Rate of mycelial spread was calculated in millimetres per 24 hr.

Large-plant tests were performed as follows: 6-week-old plants were transferred from the phytotron to a glasshouse and spray-inoculated with a water suspension of *P. tabacina* conidia (4×10^5/ml). Following inoculation, plants were kept under favourable conditions for blue mould infection and development (Cruickshank 1958). Ten days after inoculation plants were rated for disease severity (Hill and Mandryk 1962), the results being expressed as a mean plant rating for each breeding line. Ratings were based on the examination of 50 leaves of each breeding line.

The cotyledon test was performed according to Izard and Schiltz (1963), except that 50,000 conidia/ml were used in the inoculum instead of the recommended dosage of 1500 conidia/ml (Wark, Shepherd, and Mandryk, unpublished data). Strains APT1 and APT2 (Hill 1963) of *P. tabacina* were used.

III. Results

(*a*) *Uniformity of Reaction of Individual Breeding Lines*

Examination of the necrotrophic intensity of leaf disks from each of 15 plants from each breeding line after infection with mould strain APT1, showed that all

lines gave a homogeneous reaction, the coefficients of variation ranging from 2·57% for Lea A2 to 6·75% for line D-Wark. When mould strain APT2 was used, variation fell within similar limits, except for line D-Wark and GA955, where the coefficients of variation were 48·60 and 23·64%, respectively.

Results of both large-plant and cotyledon tests were similarly uniform with both mould strains, again with the exceptions of line D-Wark and GA955 following infection with mould strain APT2. Most plants in these two lines appeared highly susceptible in all tests but from test to test a varying proportion showed some resistance.

TABLE 1

COMPARISON OF THE RELATIVE RESISTANCE–SUSCEPTIBILITY STATUS OF VARIOUS TOBACCO BREEDING LINES TO BLUE MOULD STRAINS APT1 AND APT2 AS RATED BY VARIOUS METHODS

Values adjusted so that Virginia Gold = 100·0

Tobacco Breeding Line	Strain APT1			Strain APT2		
	Large-plant Test	Cotyledon Test	Combined Necrosis Rating	Large-plant Test	Cotyledon Test	Combined Necrosis Rating
N. debneyi	0	0	0·40	0	2·1	30·18
F_1 hybrid	0	0	0·06	8·7	21·1	61·74
Lea A2	0	22·2	21·08	93·9	84·2	123·22
SO1	0	22·5	25·15	82·0	94·7	122·03
Bel-12	0	27·8	54·81	61·1	63·2	115·20
GA955	81·0	55·6	81·30	116·4	78·9	121·47
Line D-Wark	107·3	44·4	85·20	115·8	84·2	128·86
Line L-Wark	4·3	28·0	38·05	94·2	36·8	119·93
Virginia Gold	100·0	100·0	100·00	100·0	100·0	100·0
EA977	0	22·2	59·40	49·2	42·1	109·11

Such results might well be found among small seed samples from a segregating population, or if incomplete gene penetrance occurred.

(*b*) *Comparison of Reaction of Different Breeding Lines*

The general appearance and course of the necrotrophic reaction have been described previously (Shepherd and Mandryk 1967). For convenience of comparison, the rating of *N. tabacum* cv. Virginia Gold (a very susceptible line) in the various tests was adjusted to 100, when either mould strain was used. With the exception of the rate of mycelial spread, the response of this line to both strains of mould was virtually identical in all tests.

Table 1 compares the assessments obtained from the large-plant tests, cotyledon tests, and the combined necrosis ratings of the various breeding lines. All three methods gave values generally low in lines regarded as "resistant" and high in lines regarded as "susceptible".

A more ready comparison of the data may be made by ranking the performances in the above tests (Table 2). Spearman's rank correlations were calculated and indicated a significant degree of correlation between the three methods.

TABLE 2

RANKING ORDER OF TOBACCO BREEDING LINES FOR RESISTANCE–SUSCEPTIBILITY STATUS TO BLUE MOULD STRAINS APT1 AND APT2

Breeding Line	Plants Infected with Strain APT1			Plants Infected with Strain APT2		
	Large-plant Test	Cotyledon Test	Combined Necrotrophy Rating	Large-plant Test	Cotyledon Test	Combined Necrotrophy Rating
N. debneyi	1	1	2	1	1	1
F_1 hybrid	1	1	1	2	2	2
Lea A2	1	3	3	6	7	9
SO1	1	5	4	5	8	8
Bel-12	1	6	6	4	5	5
GA955	8	9	8	10	6	7
Line D-Wark	10	8	9	9	8	10
Line L-Wark	7	7	5	7	3	6
Virginia Gold	9	10	10	8	10	3
EA977	1	3	7	3	4	4

Tests	Spearman Rank Correlations		Level of Significance
	Strain APT1	Strain APT2	
Large-plant × cotyledon	0·67[b]	0·70[b]	a, 0·1 –0·05
Large-plant × necrotrophy	0·60[a]	0·65[b]	b, 0·05–0·02
Cotyledon × necrotrophy	0·85[c]	0·61[a]	c, 0·01–0·001

The detailed results and the variations observed of the different tests for Virginia Gold infected with mould strain APT1 or APT2 are shown in Table 3. A

TABLE 3

COMPARISON OF RESULTS OF VARIOUS TESTS FOLLOWING INFECTION OF VIRGINIA GOLD WITH BLUE MOULD STRAINS APT1 OR APT2

Each value is the mean of results from 15 plants

Rating Method	Strain APT1	Strain APT2
Large-plant test*	3·00 ± 0·54	3·11 ± 0·56
Cotyledon test (%)†	90·00 ±24·88	95·00 ±26·26
Area with necrosis (mm^2)	90·60 ±21·24	79·60 ±18·67
Necrotrophic intensity*	2·80 ± 0·24	2·93 ± 0·25
Rate of mycelial spread (mm/24 hr)	2·12 ± 0·16	1·08 ± 0·17
Chlorophyll test (log I_0/I at 625 mμ)	0·060± 0·008	0·066± 0·009

* For details, see text.

† As percentage of seedlings on which conidial sporulation occurred.

similarity of reaction to both strains of mould validates the use of this cultivar as a comparison standard.

Actual and relative rates of spread of necrosis on the various breeding lines are shown in Table 4. Histological examination confirmed the previous suggestion by Shepherd and Mandryk (1967) that rate of spread of necrosis was directly related to rate of extension of fungal mycelium within the leaf tissues. In all cases examined, the outer edge of the mycelial border was in advance of the limit of necrosis. Comparison of the width of this outer zone (Table 4) with the data for rate of mycelial extension indicated that a visible necrotic reaction occurred some 12–24 hr after mycelial penetration of the tissues.

TABLE 4

RATE OF SPREAD OF NECROSIS IN LEAF DISKS OF BREEDING LINES INFECTED WITH BLUE MOULD STRAIN APT1 OR APT2

Breeding Line	Strain APT1		Strain APT2	
	Rate of Spread of Necrosis (mm/24 hr)	Relative Value*	Rate of Spread of Necrosis (mm/24 hr)	Relative Value*
N. debneyi	—	—	—	—
F_1 hybrid	—	—	—	—
Lea A2	—	—	3·28	303·70
SO1	—	—	3·40	314·82
Bel-12	—	—	2·80	259·26
GA955	0·50	23·58	3·08	285·18
Line D-Wark	2·04	96·23	3·30	305·56
Line L-Wark	0·18	8·49	2·94	272·22
Virginia Gold	2·12	100·00	1·08	100·00
EA977	0·20	9·43	2·32	214·81

* Values adjusted so that Virginia Gold = 100·0.

IV. DISCUSSION

The reaction of a plant to fungal infection is controlled by three major factors: the plant, genome, and the environment. To achieve an accurate measure of the innate resistance of such plants to infection by a particular fungus strain, all three factors must be held constant. However, the methods currently used to rate the resistance–susceptibility status of such plants rarely, if ever, achieve this ideal.

In the tests reported above, there was precise control of environment in both the cotyledon and necrotrophy tests, while the conditions of greenhouse growth in the large-plant test certainly fell short of the ideal. Results for all tests indicated that, with the exceptions of line D-Wark and GA955 following infection by mould strain APT2, all lines studied gave homogeneous reactions.

The genetic homogeneity of the fungus strains used in the above experiments is more difficult to assess. Plants bred for resistance to mould strain APT1 are highly selective for mould strain APT2, yet strain APT1 certainly contains a small proportion of strain APT2. Conidia of both strains are multinucleate (Shepherd 1962) and, as large conidial populations were used throughout this study, it is highly improbable

that either strain was genetically pure. Several genotypes may have been present in each strain.

A further factor influencing the resistance–susceptibility rating accorded to a particular plant variety is the actual design of the test system itself. In both the cotyledon and necrotrophy tests assessments are objective. Thus in the cotyledon test sporulation either occurs on a plant or it does not, while in the necrotrophy test necrosis either does or does not occur and actual areas are measured. In contrast, there is an element of subjectivity in assessments made by the large-plant test, where a visual estimate, subject to individual bias, is made on the lesion size and the amount of sporulation present.

In Europe the cotyledon test is widely used to predict the behaviour to blue mould infection in the field of breeding lines of tobacco. The present study was performed, *inter alia*, to examine the possibility of using the necrotrophy test to make similar predictions. Thus, with both methods, the field behaviour of plants, which is assessed by the large-plant test, is in effect the primary standard against which comparisons are made. However, in view of the considerations outlined above, we are now faced with the paradox of comparing two objective methods conducted under precisely controlled conditions with a primary standard, which is not only less precisely controlled but is somewhat subjective. Considerations such as these add weight to the recommendation of the Third World Tobacco Congress (1964) that the cotyledon test might well be used as a standard of reference for determining blue mould resistance status.

It is clear from the comparisons between resistance–susceptibility ratings shown in Table 1, that the large-plant test allows but a relatively coarse assessment of status. In their paper describing the use of the large-plant rating test, Hill and Mandryk (1962) recognized four categories only and it would appear that this test is least useful in rating the intermediate ranges of status.

From the results shown in Tables 1 and 2, it is apparent that both the cotyledon and necrotrophy tests are capable of resolving finer degrees of resistance–susceptibility status than is the large-plant test. Moreover, the correlations shown in Table 2 illustrate the good agreement between the two *in vitro* tests. The cotyledon test gives a good indication of the behaviour of a plant variety in subsequent field tests (Izard and Schiltz 1963). It is reasonable to expect, therefore, that the necrotrophy test would give a similar indication.

The cotyledon test is particularly useful in predicting the behaviour of large populations of plants and is applicable to the examination of large numbers of individuals. However, unless the individuals tested are resistant to the mould strain used, they are killed. With the necrotrophy test the plant assessed is kept free of infection and is available for future breeding work. This latter method has the additional advantage that comparisons of resistance rating among a variety of fungal strains can be made simultaneously on material from a single plant. With the cotyledon test it is clearly impossible to test single plants against a variety of fungal strains.

Hill and Green (1965) showed that, over a wide range of temperature conditions, appearance of blue mould symptoms in Virginia Gold was slower with strain APT2 than APT1. The relative rates of mycelial spread of the two mould strains would

explain this effect (Table 4). The results shown in this table indicate that Virginia Gold is the most congenial host for mould strain APT1, but not for APT2 which has the higher rate of growth in breeding lines resistant to APT1. This latter observation is in contrast to the observation by Hill and Green (1965) that blue mould symptoms take approximately the same time to develop in Virginia Gold infected with APT1 as in plants of breeding line SO1 infected with APT2. It would appear, therefore, that the time taken for gross visible symptoms to appear following infection is not a simple function of the fungal growth rate, but may be affected by the rate at which the host tissues react to infection. This suggestion would account for differences in rates of development of the necrotrophic reaction induced by a particular mould strain in various breeding lines (Shepherd and Mandryk 1967, Table 2).

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VI. References

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