Phytochemistry, 1980, Vol. 19, pp. 1057–1061. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0601-1057 $02.00/0

DIHYDROWYERONE DERIVATIVES AS COMPONENTS OF THE FURANOACETYLENIC PHYTOALEXIN RESPONSE OF TISSUES OF *VICIA FABA*

J. W. MANSFIELD*, A. E. A. PORTER† and R. V. SMALLMAN†‡

*Department of Biology and †Department of Chemistry, University of Stirling, Stirling, FK9 4LA, U.K.

(*Received* 10 *July* 1979)

Key Word Index—*Vicia faba*; Leguminosae; broad bean; *Botrytis*; phytoalexins; wyerone derivatives.

Abstract—Changes in concentrations of 7 wyerone derivatives in bean tissues undergoing resistant reactions to *Botrytis cinerea* or *B. fabae* and in cotyledons in response to mercuric chloride have been examined using high performance liquid chromatography. The proportion of derivatives occurring in their saturated (dihydro) forms varied between cotyledon, leaf and pod tissues and with time after inoculation. Unsaturated derivatives were always present in greater concentrations than their dihydro analogues.

INTRODUCTION

Tissues of broad and field bean plants (*Vicia faba* L.) produce several furanoacetylenic phytoalexins when challenged by species of *Botrytis*. Seven of the phytoalexins have been characterized as wyerone derivatives (**1–7**) [1–5]. HPLC has recently been applied to the analysis of these compounds and enabled resolution of dihydrowyerone derivatives (**4–6**) from their unsaturated analogues (**1–3**) which were not resolved by GLC, PC or TLC [2–4, 6–10].

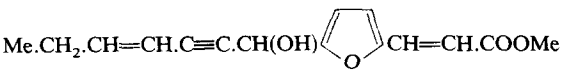
In this paper we report the use of HPLC to measure the changes in concentrations of wyerone derivatives occurring in various tissues of the broad bean plant undergoing resistant reactions to *Botrytis cinerea* Pers. ex Fr. and *B. fabae* Sard. and in cotyledons in response to the abiotic phytoalexin elicitor $HgCl_2$. Particular attention has been paid to the proportions of wyerone derivatives occurring in their dihydro forms.

RESULTS AND DISCUSSION

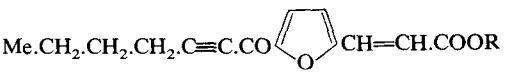
An example of a chromatogram obtained from an extract of leaf epidermis challenged with *B. cinerea* is given in Fig. 1. Wyerone derivatives were successfully resolved with repacked columns, but there were considerable differences in retention times. Retentions relative to the butyl salicylate internal standard were much less variable between columns, but in certain cases there was still overlap in relative elution times for wyerone acid and wyerone epoxide (Fig. 1). It was therefore found necessary to confirm the identity of peaks in chromatograms in any series of analyses by co-injection with authentic standards. None of the phytoalexins were detected in leaf or pod tissues before inoculation, but traces of wyerol, wyerone and their dihydro analogues were found in uninoculated cotyledons.

The most detailed studies on phytoalexin accumulation were carried out on cotyledon tissue challenged by *B. cinerea*. Wyerone and dihydrowyerone were the predominant phytoalexins in cotyledons. The proportion of the dihydro form decreased during the first 2 days after inoculation to *ca* 20% of the combined yield of the saturated and unsaturated analogues (Fig. 2). A similar decrease in the proportion of the dihydro form of wyerol was also recorded (Table 1).

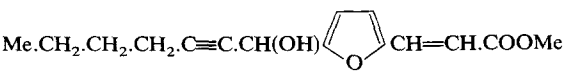
1 Wyerone, R = Me
2 Wyerone acid, R = H



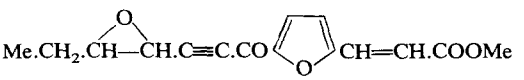
3 Wyerol



4 Dihydrowyerone, R = Me
5 Dihydrowyerone acid, R = H



6 Dihydrowyerol



7 Wyerone epoxide

‡Present address: Standardising and Analytical Department, I.C.I. Organics Division, Grangemouth, Scotland.

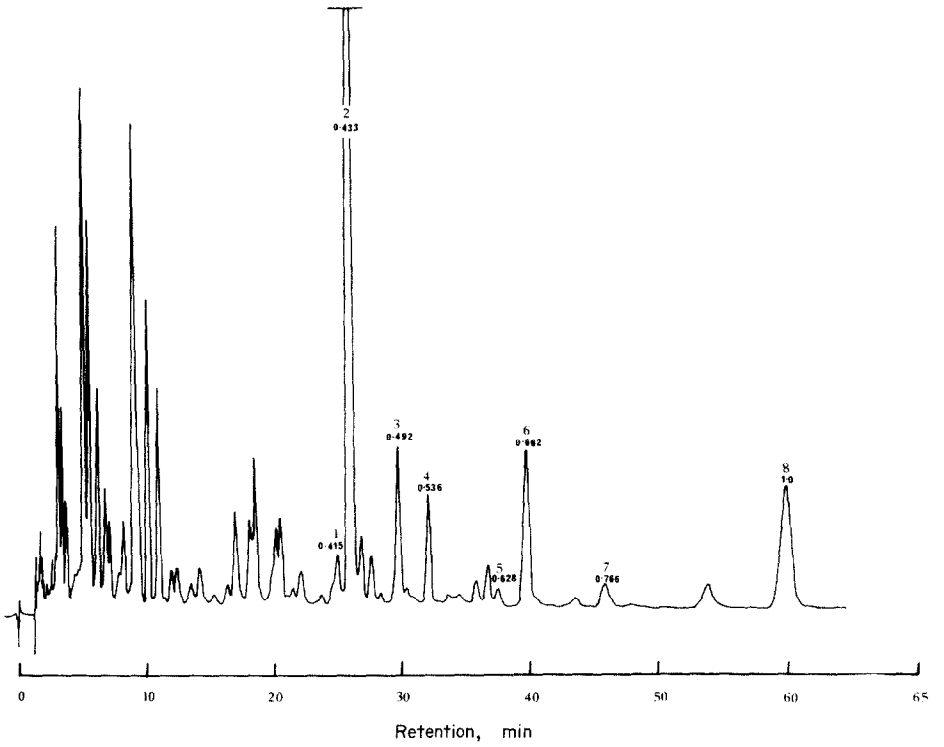


Fig. 1. Separation of wyerone derivatives from an extract of leaf epidermis collected 4 days after inoculation with *B. cinerea*; 1, wyerone epoxide; 2, wyerone acid; 3, dihydrowyerone acid; 4, wyerol; 5, dihydrowyerol; 6, wyerone; 7, dihydrowyerone; 8, *n*-butyl salicylate (internal standard). Retentions relative to 8 are given above each peak.

Wyerone acid and dihydrowyerone acid were present at much lower concentrations than the other phytoalexins and the proportion of the dihydro form of the acid was usually less than that recorded for wyerone or wyerol (Fig. 2, Table 1).

Similar patterns of phytoalexin accumulation were found in cotyledons inoculated with *Botrytis* or $HgCl_2$ (Fig. 2, Tables 1 and 2). Yields recovered after treatment with the abiotic elicitor were, however, much lower than those from tissues infected by *Botrytis*. Higher concentrations of wyerone acid and dihydrowyerone acid were found in tissues inoculated with *B. fabae* than with *B. cinerea* or $HgCl_2$, but the proportion of the dihydro analogue was closely comparable following each treatment.

Botrytis fabae, unlike *B. cinerea*, is able to spread rapidly through leaf tissue from sites inoculated with large numbers of conidia causing the development of spreading lesions [3–9]. Growth of infection hyphae of *B. fabae* is restricted, however, if inoculum concentrations are reduced to 40 or fewer conidia per 20 μl droplet and lesions produced often fail to spread from inoculation sites [9]. Changes in concentrations of wyerone derivatives in leaves were measured following inoculation with numbers of conidia of *B. cinerea* and *B. fabae* causing the development of limited lesions. Epidermal tissues, within which growth of the fungi is primarily restricted, were collected for extraction.

Wyerone acid was the major phytoalexin found in leaf epidermal tissue (Table 3). The proportions of the dihydro forms of the acid, wyerol and wyerone were typically less than those from cotyledons and again

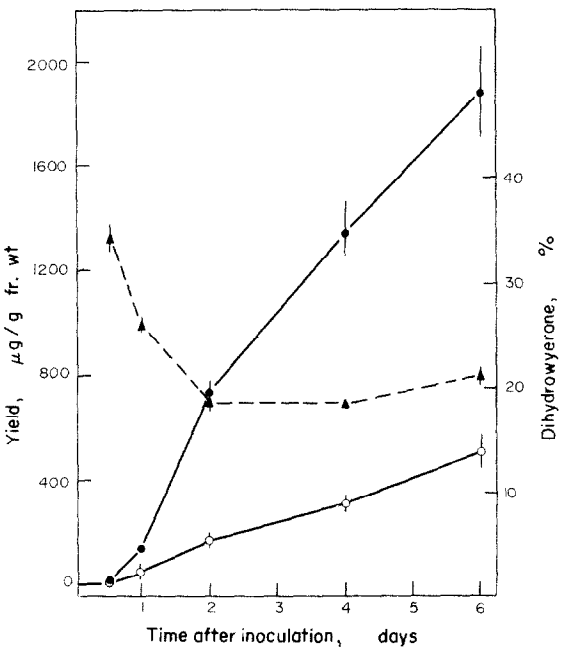


Fig. 2. Changes in the concentrations of wyerone (●—●) and dihydrowyerone (○—○) and the proportion of their combined yield occurring in the dihydro form (▲--▲) in cotyledons inoculated with *B. cinerea* (5×10^5 conidia/ml). Bars indicate range between repeated experiments using the same batch of imbibed cotyledons.

Table 1. Yields of wyerone derivatives (μg/g fr. wt) from cotyledons inoculated with *B. cinerea* (5×10^5 conidia/ml)

Wyerone derivative	Time after inoculation									
	12 hr		1 day		2 days		4 days		6 days	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Wyerone epoxide	Nd	<1.0	30.2	21.8	140.0	131.6	218.6	203.0	274.5	195.2
Wyerone acid	Nd	Nd	12.2	5.2	8.9	22.8	50.2	88.2	72.9	82.6
Dihydrowyerone acid	Nd	Nd	1.6	1.0	3.1	2.3	5.1	14.1	11.3	11.8
% Dihydrowyerone acid*	—	—	11.6	16.1	25.8	9.2	9.2	13.8	13.4	12.5
Wyerol	3.5	6.9	98.1	52.4	154.2	163.9	255.3	251.9	252.0	223.3
Dihydrowyerol	2.5	5.9	35.2	20.5	52.6	52.5	95.0	55.5	68.7	48.5
% Dihydrowyerol*	41.7	46.1	26.4	28.1	25.4	24.3	27.1	18.1	21.4	17.8

Nd = not detected. *% of combined yield of saturated and unsaturated analogues. Results from two expts using the same batch of imbibed cotyledons are given.

decreased with time after inoculation. The higher yields of phytoalexins recovered from leaves inoculated with *B. cinerea* than with *B. fabae* were associated with more extensive necrosis of the tissue in response to the higher numbers of conidia of the former species within inoculum droplets.

Results of HPLC analyses carried out on pod endocarp tissue inoculated with *B. cinerea* are reported in Table 4. Yields of the wyerone derivatives recovered were lower from pods than cotyledon or leaf epidermal tissues and, as in leaves, wyerone acid reached the highest concentration. The proportions of dihydro derivatives were intermediate between those found in cotyledons and leaves challenged by *B. cinerea*.

In general, results of HPLC analyses confirmed those obtained by TLC/UV methods to measure phytoalexin accumulation. Wyerone and wyerone acid were the predominant phytoalexins in cotyledons, and leaves and pods respectively, and the inhibitors accumulated, rapidly during the formation of limited lesions [3, 8–10]. However, it must be emphasised that yields reported for wyerol, wyerone and wyerone acid in earlier studies performed without HPLC comprised varying proportions of their corresponding dihydro derivatives.

The biogenetic relationship between the saturated and unsaturated analogues remains to be determined. The simple scheme proposed [3], wyerol → wyerone → wyerone acid, may also be applied to their

Table 2. Yields of wyerone derivatives (μg/g fr. wt) from cotyledons inoculated with *B. fabae* (5×10^5 conidia/ml) or $HgCl_2$ (10^{-2}M)

Wyerone derivative	Time after inoculation					
	12 hr		1 day		4 days	
	Expt 1	Expt 2	Expt 1	Expt2	Expt 1	Expt 2
(a) *B. fabae*						
Wyerone epoxide	Nd	1.0	36.7	28.4	196.7	160.3
Wyerone acid	Nd	Nd	33.4	31.2	257.3	288.7
Dihydrowyerone acid	Nd	Nd	6.0	2.5	12.8	24.3
% Dihydrowyerone acid*	—	—	15.2	7.4	4.7	7.8
Wyerol	4.5	1.8	111.6	99.1	163.7	253.8
Dihydrowyerol	2.1	1.1	34.3	29.9	24.5	61.0
% Dihydrowyerol*	31.8	37.9	23.5	23.2	13.0	19.4
Wyerone	2.4	1.7	217.1	190.2	1065.0	924.7
Dihydrowyerone	1.2	<1.0	56.9	48.6	170.2	184.6
% Dihydrowyerone*	33.3	—	20.8	20.4	13.8	16.6
(b) $HgCl_2$						
Wyerone epoxide	1.8	1.9	6.0	18.5	52.6	44.2
Wyerone acid	Nd	Nd	<1.0	<1.0	80.0	55.8
Dihydrowyerone acid	Nd	Nd	Nd	Nd	5.3	6.1
% Dihydrowerone acid*	—	—	—	—	6.2	9.9
Wyerol	1.5	2.0	8.1	11.7	42.7	35.4
Dihydrowyerol	1.0	1.6	7.4	7.5	11.0	9.4
% Dihydrowyerol*	40.0	44.4	47.7	39.1	20.5	21.0
Wyerone	3.2	5.5	35.2	49.3	580.6	493.7
Dihydrowyerone	1.6	2.8	14.0	17.2	144.2	126.2
% Dihydrowyerone*	33.3	33.7	28.5	25.9	19.9	20.4

*% of combined yield of saturated and unsaturated analogues. Results from two expts using the same batch of imbibed cotyledons are given.

Table 3. Yields of wyerone derivatives (μg/g fr. wt) from epidermal tissue in leaves undergoing resistant reactions to *B. cinerea* or *B. fabae* (*ca* 2000 and *ca* 40 conidia/20 μl inoculum droplet, respectively)

Wyerone derivative	Time after inoculation					
	12 hr		1 day		4 days	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
(a) *B. cinerea*						
Wyerone epoxide	2.3	Nd	10.3	13.4	10.4	16.9
Wyerone acid	20.2	18.8	262.1	171.3	610.3	473.1
Dihydrowyerone acid	4.3	3.0	19.1	16.4	41.0	29.5
% Dihydrowyerone acid*	17.6	13.8	6.8	8.7	6.3	5.9
Wyerol	6.4	3.4	18.9	27.7	4.5	30.4
Dihydrowyerol	4.3	2.2	<1.0	10.3	Nd	5.0
% Dihydrowyerol*	40.2	39.3	—	27.1	—	14.1
Wyerone	7.9	4.6	88.8	25.5	53.2	78.0
Dihydrowyerone	Nd	<1.0	12.4	5.0	4.5	9.0
% Dihydrowyerone*	—	–	12.3	16.4	7.8	10.3
(b) *B. fabae*						
Wyerone epoxide	Nd	Nd	11.5	4.6	48.4	29.7
Wyerone acid	10.5	10.3	108.2	52.7	229.6	123.3
Dihydrowyerone acid	2.3	4.0	13.8	3.5	17.6	3.2
% Dihydrowyerone acid*	18.0	28.0	11.3	6.2	7.1	2.5
Wyerol	14.1	2.9	6.3	12.9	40.6	17.0
Dihydrowyerol	13.1	<1.0	<1.0	<1.0	4.2	3.9
% Dihydrowyerol*	48.2	—	—	—	9.4	18.7
Wyerone	2.4	<1.0	15.4	7.8	67.4	63.6
Dihydrowyerone	1.4	Nd	1.4	<1.0	5.0	6.9
% Dihydrowyerone*	36.8	—	8.3	—	6.9	9.8

* % of combined yield of saturated and unsaturated analogues. Results from two expts are given.

dihydro derivatives. The observed differences in proportions of dihydro forms (being highest) with wyerol and lowest with wyerone acid) and also the decreasing proportions of dihydro compounds with time after inoculation may be explained by the biosynthetic steps involving dihydro derivatives taking place at a slower rate than those of their unsaturated analogues.

The finding that $HgCl_2$ elicits synthesis of the furanoacetylenic phytoalexins parallels the reported accumulation of isoflavonoid and sesquiterpenoid phytoalexins following treatment with salts of mercury and other heavy metals and provides support for the proposal that phytoalexins may be considered a special class of stress compounds [11–14]. The precise mechanisms by which microorganisms and abiotic elicitors stimulate phytoalexin biosynthesis is not clear.

Table 4. Yields of wyerone derivatives (μg/g fr. wt) from pod endocarp tissue inoculated with *B. cinerea* (5×10^5 conidia/ml)

Wyerone derivative	Time after inoculation					
	12 hr		1 day		4 days	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Wyerone epoxide	Nd	<1.0	8.0	6.1	16.8	18.4
Wyerone acid	3.1	3.0	28.1	25.0	85.2	118.3
Dihydrowyerone acid	1.1	<1.0	4.2	3.7	9.0	14.7
% Dihydrowyerone acid	26.2	—	13.0	12.9	9.6	11.1
Wyerol	<1.0	Nd	4.5	7.2	2.6	1.8
Dihydrowyerol	<1.0	Nd	1.3	2.4	Nd	<1.0
% Dihydrowyerol*	—	—	22.4	25.0	—	—
Wyerone	Nd	Nd	3.3	6.4	20.7	56.0
Dihydrowyerone	Nd	Nd	<1.0	2.2	3.7	9.0
% Dihydrowyerone*	—	—	—	25.6	15.2	13.8

* % of combined yield of saturated and unsaturated analogues. Results from two expts are given.

The $HgCl_2$ treatment used in this work caused the death of many cells beneath inoculum droplets by 12 hr after inoculation (as indicated by uptake of 1% aqueous Evans blue stain). Phytoalexin production following inoculation with *Botrytis* is also associated with the early death of cells following fungal penetration into the epidermis [9, 10]. It is possible, therefore, that compounds released from dead or dying cells were the major causes of phytoalexin biosynthesis by surrounding living tissues rather than fungal metabolites or $HgCl_2$ directly inducing the synthesis of enzymes for furanoacetylenic biosynthesis [10–14].

Results of preliminary experiments suggest that the dihydro derivatives and their unsaturated analogues possess similar antifungal activities against *Botrytis* ([1] and unpublished observations). Earlier data in which yields of mixtures of unsaturated and saturated derivatives were recorded therefore probably provided a good indication of the potential significance of phytoalexins in causing the restriction of fungal growth within infected tissues [2–4, 6, 8–10]. It is clear, however, that the occurrence of dihydro derivatives should no longer be overlooked, particularly when considering the biogenesis of the phytoalexins and the factors eliciting their synthesis.

EXPERIMENTAL

Isolates of *B. cinerea* (Herb. IMI 225854) and *B. fabae* (Herb. IMI 225851) were those used in previous studies [3–5, 15]. Sporulating cultures were produced in flasks containing medium X [16] and suspensions of conidia in sterile dist. H_2O prepared as previously described [15, 17]. Spore concns were determined from haemocytometer counts and adjusted as required. The broad bean cv Aquadulce was used throughout the work. Imbibed cotyledons, detached field-grown leaves and pods were prepared for inoculation as described elsewhere [3, 4, 18]. Cotyledons and leaves were inoculated with *ca* 20 μl droplets of spore suspensions or $HgCl_2$ soln (0.01 M aqueous). Droplet size on pod endocarp varied depending on the vol. required to cover the surface of seed cavities. Inoculated tissues were incubated at 18° either in the dark (cotyledons and pods) or illuminated for 16 hr each day in a growth cabinet. Cotyledon and pod tissues were collected for extraction as described in ref. [3] except that following treatment with $HgCl_2$, in addition to tissue below inoculum droplets, a surrounding narrow band of tissue ($\leq$ 2 mm in width) was also collected as it developed an intense brick red colouration between 1 and 4 days after inoculation. Tissue beneath $HgCl_2$ droplets became grey after 1 day. Epidermis (contaminated with some mesophyll) was stripped from at least 200 inoculation sites on leaves at each time allowing the collection of at least 0.2 g fr. wt for extraction. Cotyledon and pod tissue samples weighed *ca* 3 and 5 g respectively. The times after inoculation at which samples were taken represent the mid point of the collection period. All tissues were stored at −20° before extraction with Et_2O [3, 10]. Extracts of cotyledons and pods were taken to dryness *in vacuo* at 30° and the residue resuspended in an appropriate vol. of MeOH (1 ml MeOH per 0.2–8 g original tissue) containing 3 mg/ ml *n*-butyl salicylate int. standard. For leaf extracts the standard was added to the Et_2O soln before evapn and the combined residue resuspended in MeOH without additional butyl salicylate. Reverse phase HPLC was performed as described in detail elsewhere [7] using a solvent programmer. Aliquots (10 μl) of MeOH solns were injected on to a 20 cm × 8 mm id column packed with ODS Hypersil (Shandon Southern Ltd.) and fitted with an on-column needle through septum injector. Gradient elution was carried out at 30° with a flow rate of 5 ml/min and UV detection was at 330 nm (0.1 *A* units for full scale deflection). Initial solvent conditions were MeOH–5% HCO_2H (7:13) running linearly over 30 min to final conditions MeOH–MeCN–5% HCO_2H (4:1:5). Peak areas were measured using a computing integrator and phytoalexin yields calculated from the calibration equations derived for μg phytoalexin injected and ratio of peak area to that of the int. standard [7]. An integrator response of 3000 units was considered the limit of quantitative detection. Deterioration of column performance following damage during injection necessitated column repacking during the work.

Acknowledgements—The financial support of RVS by Imperial Chemical Industries Ltd., Organics Division, and the technical assistance of D. P. Donnelly are gratefully acknowledged.

REFERENCES

1. Fawcett, C. H., Spencer, D. M., Wain, R. L., Fallis, A. G., Jones, E. R. H., Le Quan, M., Page, C. B., Thaller, V., Shubrook, D. C. and Whitham, P. (1968) *J. Chem. Soc. C* 2455.
2. Letcher, R. M., Widdowson, D. A., Deverall, B. J. and Mansfield, J. W. (1970) *Phytochemistry* **9**, 249.
3. Hargreaves, J. A., Mansfield, J. W. and Rossall, S. (1977) *Physiol. Plant Pathol.* **11**, 227.
4. Hargreaves, J. A., Mansfield, J. W. and Coxon, D. T. (1976) *Phytochemistry* **15**, 651.
5. Hargreaves, J. A., Mansfield, J. W., Coxon, D. T. and Price, K. R. (1976) *Phytochemistry* **15**, 1119.
6. Fawcett, C. H., Firn, R. D. and Spencer, D. M. (1971) *Physiol. Plant Pathol.* **1**, 163.
7. Smallman, R. V., Porter, A. E. A. and Mansfield, J. W. (1979) *J. Chromatogr.* **172**, 498.
8. Mansfield, J. W. and Deverall, B. J. (1974) *Ann. Appl. Biol.* **77**, 227.
9. Mansfield, J. W. (1980) in *The Biology of* Botrytis (Coley-Smith, J. R., Jarvis, W. R. and Verhoeff, K., eds.) in press. Academic Press, London.
10. Mansfield, J. W., Hargreaves, J. A. and Boyle, F. C. (1974) *Nature* **252**, 316.
11. VanEtten, H. D. and Pueppke, S. G. (1976) in *Biochemical Aspects of Plant–Parasite Relationships* (Friend, J. and Threlfall, D. R., eds.). Academic Press, London.
12. Stoessl, A., Stothers, J. B. and Ward, E. W. B. (1976) *Phytochemistry* **15**, 855.
13. Cheema, A. S. and Haard, N. F. (1978) *Physiol. Plant Pathol.* **13**, 233.
14. Hargreaves, J. A. (1979) *Physiol. Plant Pathol.* **15**, 279.
15. Rossall, S., Mansfield, J. W. and Hutson, R. A. (1980) *Physiol. Plant Pathol.* (in press).
16. Last, F. T. and Hamley, R. E. (1956) *Ann. Appl. Biol.* **44**, 410.
17. Balasubramani, K. A., Deverall, B. J. and Murphy, J. V. (1971) *Physiol. Plant Pathol.* **1**, 105.
18. Purkayastha, R. P. and Deverall, B. J. (1965) *Ann. Appl. Biol.* **56**, 269.