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AMMONIA-LYASE AND *O*-METHYL TRANSFERASE ACTIVITIES RELATED TO LIGNIFICATION IN WHEAT LEAVES INFECTED WITH BOTRYTIS

ANDREW J. MAULE and JONATHAN P. RIDE
Department of Microbiology, The University of Birmingham, Birmingham B15 2TT, England

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; *Botrytis cinerea*; fungi; lignification; control; phenylalanine ammonia-lyase; tyrosine ammonia-lyase; caffeic acid *O*-methyl transferase; 5-hydroxyferulic acid *O*-methyl transferase.

Abstract—The deposition of lignin around wounds in wheat leaves infected with the non-pathogenic fungus *Botrytis cinerea*, was preceded by increases in ammonia-lyase and *O*-methyl transferase activities. The increases were localised in the lignifying tissues. Phenylalanine ammonia-lyase and tyrosine ammonia-lyase increased concurrently and were inseparable by gel filtration or DEAE cellulose chromatography, the two activities probably being the function of a single protein. Infection-specific isoenzymes for either of the ammonia-lyase activities were absent. Increases in caffeic acid *O*-methyl transferase and 5-hydroxyferulic acid *O*-methyl transferase activities closely followed those of the ammonia-lyases. It is unlikely that the increased proportion of syringyl groups found in infection-induced lignin is controlled by *O*-methyl transferase activity. A modified method for the estimation of *O*-methyl transferase activity in crude wheat extracts is described.

INTRODUCTION

Lignification as a response to fungal infection has been demonstrated in a variety of hosts [1–3]. Recent work has provided evidence that the deposition of lignin in tissues surrounding the areas of growth of the non-pathogenic fungus *Botrytis cinerea*, in wounds on the primary leaves of wheat, functions as a physical barrier to further invasion of the host [4]. The induced lignin contained a much higher proportion of syringyl to guaiacyl residues than that extracted from healthy tissues. A detailed study of the metabolic changes associated with this phenomenon could yield information concerning factors controlling the lignin biosynthetic pathway as well as further insight into lignification as part of the hypersensitive response to fungal infection.

Phenylalanine ammonia-lyase (PAL), EC.4.3.1.5. and tyrosine ammonia-lyase (TAL), EC.4.3.1.5. were studied. These are the first enzymes unique to the phenolic pathway and PAL has been implicated as a control enzyme [5]. Caffeic acid *O*-methyl transferase (CA-*O*MT), EC.2.1.1.6. and 5-hydroxyferulic acid *O*-methyl transferase (5FHA-*O*MT) EC.2.1.1.6. were also studied, since the enzymes have been implicated in a control mechanism for lignin structure [6–8], in which feedback inhibition by 5HFA on CA-*O*MT also occurs [7].

RESULTS AND DISCUSSION

Ammonia-lyase activities

PAL and TAL activities in buffered extracts of acetone powders were only 30% of the maximum obtainable by incubation of the complete acetone powders in buffered substrates. The absence of activity in a cell wall fraction prepared from fresh tissue indicated that the retention of activity in the powder was not due to wall-association but other factors, one of which may be incomplete maceration of the tissue in the preparation of the acetone precipitates.

Time course studies for PAL and TAL (Figs. 1 and 2) showed a significant ($p < 0.01$) increase in activity over the uninfected control at 18 hr with some increase earlier, 12 hr after inoculation. This is in agreement with the hypothesis that increases in early enzymes must occur before accumulation of the end-product; lignin being present at 18 hr, complete encircling of the infected site occurring by 24 hr [4]. Excision of infected wounds together with immediately adjacent tissue revealed that more than 95% of the increases in PAL and TAL activity were confined to an area extending not more than 0.5 mm from the wound (Table 1). Hence the increases are localised in the tissues undergoing lignification. A similar correlation between lignin formation and PAL activity has been demonstrated in asparagus [9]. In bamboo a correlation has been shown for the activities of both PAL and *O*MT [10,11].

As early enzymes in the phenolic pathway PAL and TAL catalyse similar deamination reactions. This and the fact that in some preparations PAL and TAL activities are closely associated has led to some uncertainty as to the exact relationship between the two [5], although it has been suggested that TAL activity is a consequence of low substrate specificity exhibited by the PAL enzyme [12]. In an attempt to clarify the relationship between PAL and TAL in wheat, extracts of acetone powders were subjected to column chromatography on DEAE cellulose and Sepharose 4B. It was hoped that these methods would determine whether PAL or TAL exist in different forms and whether fungal infection specifically stimulates a particular isoenzyme or induces

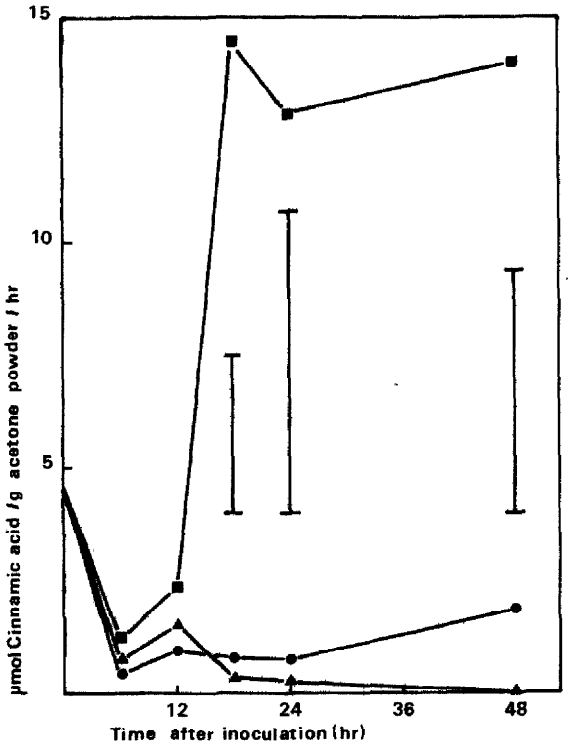

Fig. 1. Changes in PAL activity in healthy[a] (▲——▲), control[b] (●——●) and inoculated (■——■) leaves, with time. Each point is the mean of three tissue samples. Bars represent LSD ($p < 0.01$). [a]Unwounded, untreated. [b]Wounded, treated with water.

new isoenzymes. The occurrence of PAL isoenzymes with differing properties has been shown in sweet potato [13], oak leaves [14] and in mung bean seedlings stimulated by red light [15].

The elution profiles from both types of column show a single major peak for both PAL and TAL activities eluted at an ionic strength equivalent to 0.13 M NaCl and corresponding to a MW of *ca* 400000. In all cases TAL activity in fractions associated with the major peak did not deviate significantly from 11% of PAL activity. This ratio was not upheld when TAL activity was very low, probably due to the difficulty in accurately measuring such activity, although PAL activity was always greater than that of TAL. These results are consistent with the hypothesis that PAL and TAL activities are functions of the same protein. This is contrary to the conclusions of Young and Neish [16] but in agreement with those of Nari *et al.* [17]. Two minor peaks of lower MW were separated on Sepharose 4B and were possibly subunits of the larger MW species [17]. No new isoenzymes could be detected following fungal infection.

O-*Methyl transferase activities*

TLC analysis of the ^{14}C-labelled reaction products from the *O*MT assay of Glass and Bohm [18] using a crude enzyme preparation, showed a partition of the label into at least two fractions; the expected product and a fraction positioned close to the origin. This was particularly evident in the case of CA-*O*MT. The presence of labelled compounds with high polarity (low R_f), suggested that the normal product was undergoing a further reaction, e.g. ferulic acid (FA) to FA-X. Such a distribution of label was not observed in the no-substrate controls used in the assay. TLC analysis of the ether

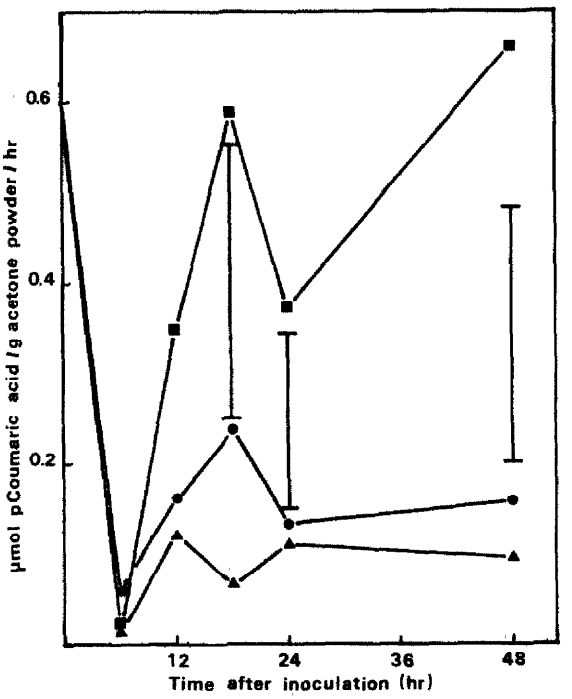

Fig. 2. Changes in TAL activity in healthy[a] (▲——▲), control[b] (●——●) and inoculated (■——■) leaves, with time. Each point is the mean of three tissue samples. Bars represent LSD ($p < 0.01$). [a]Unwounded, untreated. [b]Wounded, treated with water.

Table 1. Localisation of enzyme activities in tissues surrounding wheat leaf wounds infected with *B. cinerea*

	PAL Activity µmol Cinnamic acid/g acetone powder /hr	TAL Activity µmol *p*-Coumaric acid/g acetone powder /hr	CA–OMT Activity nmol Ferulic acid /mg Protein/hr	5HFA–OMT Activity nmol Sinapic acid /mg protein/hr
Untreated leaves.	0.25	0.05	6.15	8.48
Leaf remains after removal of infected wounds.	0.37	0.14	7.44	8.17
Isolated infected wounds*	16.5	2.95	25.9	25.7

* Wounded, infected tissue plus an average of 0.5 mm "healthy" tissue surrounding it.

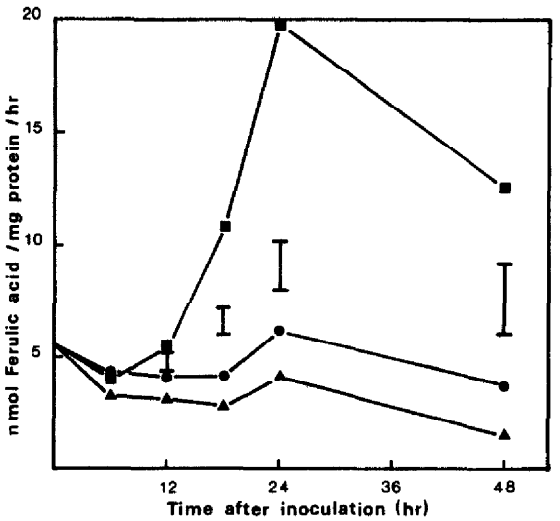

Fig. 3. Changes in CA-OMT activity in healthy [a](▲——▲), control[b] (●——●) and inoculated (■——■) leaves, with time. Each point is the mean of three tissue samples. Bars represent LSD ($p < 0.01$). [a]Unwounded, untreated. [b]Wounded, treated with water.

extract after treatment of the enzyme digest with M alkali for 20 hr, *in vacuo*, prior to acidification showed a decrease in the counts present at low R_f and a corresponding increase in the counts associated with the *O*MT product. These provided 93–97% of the total counts present in the ether extract. This high proportion of label present as product in the unchromatographed ether extract justifies the absence of a chromatographic separation from the routine assay. The improved recovery of product from the aqueous phase by alkali pre-treatment when estimating activity in crude extracts improves the accuracy of the assay. The modified assay procedure did not result in any deviation from pH 8 as the optimum for activity measured with untreated digests [11].

As with PAL and TAL, time course studies for CA-*O*MT and 5HFA-*O*MT showed an increase in infected tissues over the uninfected control at 12 hr (Figs. 3 and 4). The increase was localised in tissue immediately surrounding the infected area (Table 1). It is noteworthy that over the period of rapid lignification, 18 to 24 hr [4], the ratio of CA-*O*MT to 5HFA-*O*MT does not deviate significantly from 1.1:1, the ratio found in healthy leaves. This is in good agreement with the ratio measured by Kuroda *et al.* [8]. Hence it appears that CA-*O*MT and 5HFA-*O*MT whether as two enzymes or as activities of the same protein [7], do not function as control points for the modified structure of the induced lignin. Future studies on the remaining steps in the pathway should provide a clearer picture as to the metabolic alterations associated with the increased syringyl/guaiacyl ratio resulting from fungal infection.

The increase in PAL, TAL, CA-*O*MT and 5HFA-*O*MT activities upon wounding alone is not unexpected as wounds are open to infection by any of the leaf surface microflora, many of which may stimulate enzymes of the phenolic pathway to a lesser extent. Increases in PAL activity associated with tissue damage have been reported [13,19]. The increases found upon wounding are, however, small compared to those induced by fungal infection. The good correlation that exists both spatially and temporally between lignification and ammonia-lyase or *O*MT activity suggests that increased levels of the enzymes are an important part of the defence mechanisms of the plant, although the mechanism by which the levels are stimulated by the invading fungus is not yet clear.

Good evidence that the increases in activities shown are due to stimulation of the host, but not of fungal enzymes, is provided by the absence of activity in mycelium and culture filtrates of *B. cinerea* grown on wheat extract medium.

EXPERIMENTAL

Materials. 5-Hydroxyferulic acid was synthesised from 5-hydroxyvanillin and malonic acid [20]. 5-Hydroxyvanillin was prepared from 5-iodovanillin [21]. *S*-Adenosylmethionine-[^{14}C-CH$_3$](SAM) was obtained from the Radiochemical Centre, Amersham.

Plant and fungal material. The maintenance of cultures, production of spore suspensions of *B. cinerea* and production and inoculation of 7-day-old seedlings of *Triticum aestivum* cv Joss Cambier were as previously described [4].

Enzyme preparations. All procedures were carried out at 0–4° unless otherwise stated. For the measurement of ammonia-lyase activity Me_2CO powders were prepared from 6 cm sections (10 wounds) of primary leaves according to ref. [22]. For the preparation of a cell wall fraction, 6 cm sections were ground in a mortar and pestle with 0.1 M Pi buffer pH 6 to a cell free extract. The extract was filtered and the solids washed with 10 to 12 vol of buffer. Me_2CO powders were prepared from the solids as before. For the *O*MT assays, 6 cm sections of primary leaves were ground to a cell free extract with ×0.1 wt Polyclar AT and ground glass in 0.1 M Tris–HCl buffer pH 8. The extract was centrifuged at 2000 *g* for 10 min to remove the gross solids. This was followed by a centrifugation of the supernatant at 35000 *g* for 1 hr, the supernatant being used as the enzyme preparation. For the assay of fungal protein for *O*MT and ammonia-lyase activity, flasks of wheat extract medium were inoculated with 0.5 ml of a spore suspension prepared as before [4], the mycelium and culture filtrate being harvested after 48 hr at 25° and prepared as for the plant material for the appropriate assay.

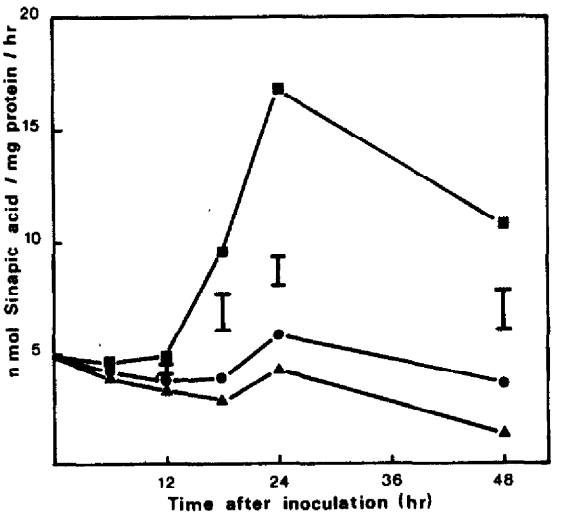

Fig. 4. Changes in 5HFA-OMT activity in healthy [a](▲——▲), control[b] (●——●) and inoculated (■——■) leaves, with time. Each point is the mean of three tissue samples. Bars represent LSD ($p < 0.01$). [a]Unwounded, untreated. [b]Wounded, treated with water.

Wheat extract medium was prepared by homogenising wheat leaves with ×10 wt H_2O followed by filtration. The filtrate was adjusted to pH 3 with conc HCl and centrifuged at 10000 *g* for 20 min. After readjustment to pH 6 with 4N NaOH, the soln was recentrifuged and the supernatant sterilised by filtration.

Enzymes assays. PAL was assayed using a spectrophotometric method similar to that of ref. [23]. A known wt (10–20 mg) of Me_2CO powder was incubated with 3 ml 33 mM L-phenylalanine in 0.1 M Na_2 borate buffer pH 8.8 in small screw cap bottles at 40° for 2 hr with continual shaking. The reaction was stopped by the addition of 0.2 ml 5 M HCl. After filtration solids were washed twice with 3 ml dist H_2O and the pooled filtrates extracted twice with 1 vol Et_2O. Et_2O was removed by evaporation *in vacuo* at 25°, the residue being dissolved in 3 ml 50 mM NaOH. The soln was acidified with 0.2 ml 1.5 M HCl and the A at 278 nm recorded. TAL was assayed similarly; the Me_2CO powder was incubated with 3 ml 1.84 mM L-tyrosine in 0.1 M Na_2 borate buffer pH 8.8 at 40° for 3 hr. The reaction was stopped by addition of 0.2 ml 5 M HCl and the digest extracted as above. After removal of the Et_2O the residue was dissolved in 3 ml 50 mM NaOH and the A at 333 nm recorded. For the assay of column eluates, 1 ml of enzyme soln was incubated with 2 ml buffered substrate (0.15 M L-phenylalanine or 2.76 mM L-tyrosine) and extracted as above. No-substrate controls were run in duplicate in each case. *O*MT assays were carried out using a method based on that of ref. [18]. 0.5 ml of a soln of 5 mM substrate (caffeic acid or 5-hydroxyferulic acid) and 8 mM ascorbic acid, 0.5 ml of 2 mM $MgCl_2$ in 0.1 M Tris–HCl buffer pH 8 and 1 ml of 1 mM *S*-adenosylmethionine-[^{14}C-Me] (50 μCi/mmol) were equilibrated together at 37° for 5 min, 1 ml of enzyme was added and the whole incubated at 37° for 30 min. The reaction was stopped by the addition of 1 ml 4 M NaOH. After mixing the reaction tubes were stoppered and left *in vacuo* at room temp for 20 hr. 1.3 ml of 5 M HCl were added and the digests extracted twice with 1 vol Et_2O. The organic phase was transferred to a scintillation vial and evaporated to dryness under a stream of air at room temp. 10 ml of toluene based scintillant were added and the radioactivity counted. To test the effectiveness of alkali treatment prior to Et_2O extraction, the reaction in control samples was stopped by the addition of 0.2 ml 5 M HCl and the digest extracted immediately. Protein concn in the enzyme preparations was determined by the method of ref. [24].

Column chromatography. Me_2CO powders from healthy and infected material were extracted with 50 mM Na_2 borate buffer pH 8.8 (1 ml to 1 mg), with an equal wt of Polyclar AT, at 0° for 1 hr. The extract was filtered and the filtrate freeze-dried. Dried extracts were dissolved in a minimum quantity of H_2O and loaded onto Sephadex G25 "fine". The column was eluted with dist H_2O and the high MW fraction immediately freeze-dried. For DEAE cellulose separation the freeze-dried sample was dissolved in a minimum of 50 mM Tris–HCl buffer pH 8.2. Proteins were eluted from the column with a linearly increasing gradient of NaCl in the same buffer at 30 ml/hr. For Sepharose 4B separation the freeze-dried sample was dissolved in a minimum of 20 mM Na_2 borate buffer pH 8.8 and eluted from the column with the same buffer at 16 ml/hr. Calibration of this column for the determination of MW was achieved by running standard proteins (ovalbumin; bovine serum albumin; alcohol dehydrogenase [yeast]; urease [jack-bean] and thyroglobulin [hog]) through the same column. All columns were run at 0–4°.

TLC. After evaporation of Et_2O from extracts of enzyme digests the residues were dissolved in a min vol of Me_2CO (30–40 μl) and applied to a Si gel plate. The plates were developed for 16 cm in C_6H_6–HOAc–H_2O (10:7:3, upper phase). Sections (1 cm) of Si gel were scraped from the plates into centrifuge tubes. Labelled compounds were eluted twice with Me_2CO (3 ml), the gel being centrifuged between washings. The supernatants were pooled in scintillation vials and the Me_2CO removed under a stream of air at room temp, 10 ml of toluene based scintillant were added and the radioactivity counted.

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