*Phytochemistry*, Vol. 26, No. 10, pp. 2723–2727, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00


# THE ESTIMATION OF L-PHENYLALANINE AMMONIA-LYASE SHOWS PHENYLPROPANOID BIOSYNTHESIS TO BE REGULATED BY L-PHENYLALANINE SUPPLY AND AVAILABILITY

ANTOINETTE DA CUNHA†

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RA, U.K.

(*Received* 16 *February* 1987)

**Key Word Index**—*Phaseolus vulgaris*; Leguminosae; *Solanum tuberosum*; Solanaceae; phenylpropanoid biosynthesis; protein synthesis; L-phenylalanine ammonia-lyase; L-phenylalanine amino-transferase.

**Abstract**—L-Phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) is the first committed enzyme in the pathway leading to phenylpropanoid biosynthesis in higher plants. PAL catalyses the conversion of L-phenylalanine to *t*-cinnamic acid with the elimination of ammonia. Standard methods for determination of PAL activity in both green and non-green tissues were found to lead to measurements of both L-phenylalanine amino-transferase (PAT, E.C. 2.6.1.1) and PAL activities together. The accurate estimation of PAL activity alone, necessitated the inhibition of PAT by a specific inhibitor of PAT activity, L-aspartic acid. The influence of PAT on the kinetics of PAL activity may explain (i) the diverse properties that have been attributed to PAL and (ii) the controversies regarding the control mechanism underlying the regulation of PAL activity. Evidence is presented for the regulation of phenylpropanoid biosynthesis via substrate supply and availability as opposed to feedback inhibition, during phaseollin production and hypersensitive necrosis in *Phaseolus vulgaris*.

## RESULTS

### *The spectrophotometric assay*

PAL catalyses the conversion of L-phenylalanine to *t*-cinnamic acid with the elimination of ammonia. The *t*-cinnamic acid produced is commonly measured spectrophotometrically. Times of incubation ranged from 0 to 22 hours [1–3]. Attempts were made to estimate PAL activities using the spectrophotometric assay in green and non-green tissue homogenates. Systems used were bean, pea, maize hypocotyls and leaves, potato tuber tissue and bean hypocotyl-derived callus and cell suspension cultures. Results on PAL activities were non-linear with respect to time and protein concentration. Partial purification by gel filtration did not result in linearity. Non-linearity with time and protein concentration could have been due to (i) unreliable estimation of protein, (ii) microbial contamination or (iii) absorption of compounds other than *t*-cinnamic acid at these wavelengths.

The estimation of protein after removal of pigments by acetone precipitation [4] and by the method of ref. [5] and the use of shorter periods of incubation using sterile water, ruled out possibilities (i) and (ii). Possibility (iii) could be attributed to the absorption of (a) keto-enol-borate complexes produced from endogenous keto acids in presence of borate buffer [6] and/or (b) other phenolic compounds. Since both sample and reference cuvettes contained similar constitutents (D-phenylalanine substituting L-phenylalanine in reference cuvettes), and substitution of borate buffer by other buffers (e.g. Tris) did not result in linearity, possibility 3(a) was ruled out. Possibility 3(b) could be attributed to the absorption of phenolic compounds other than *t*-cinnamic acid produced from L-phenylalanine (Fig. 1). Of these compounds, L-phenylpyruvic acid was found to exhibit an absorption spectrum similar to *t*-cinnamic acid (in 0.1 M sodium borate buffer, pH 8.8, both *t*-cinnamic acid and L-phenylpyruvic acid were found to have similar molar absorption coefficients, $\lambda_{max}^{Na_2B_4O_7}$ nm (log E): 290 (2.32), 265 (2.30).

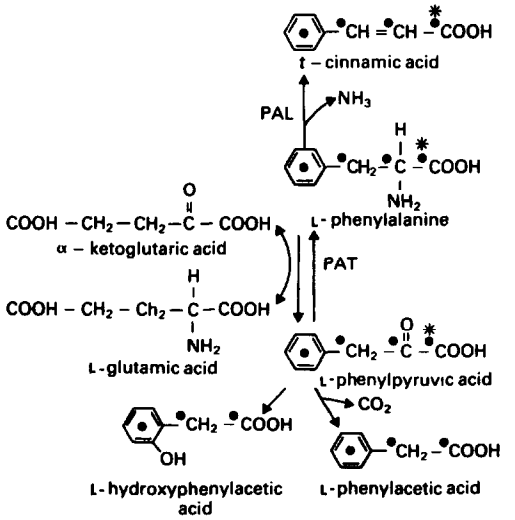


Fig. 1. The route of L-phenylalanine, [U-$^{14}$C]-L-phenylalanine (●) and [1-$^{14}$C]-L-phenylalanine (✳) during the spectrophotometric and isotopic assays for PAL activity.

† Present address: Department of Microbiology and Public Health, Michigan, State University, East Lansing, MI 48824, U.S.A.

*The* L-[U-$^{14}$C]*phenylalanine isotopic assay*

Isotopic measurements of *t*-cinnamic acid using uniformly labelled L-phenylalanine as substrate [1, 7] also yielded PAL activities non-linear with time and protein concentration. Using this alternative method for measuring PAL activity, Wilkinson [7] also reported similar findings in different plant systems. The reasons for non-linearity with time and protein concentration could have been due to the incorporation of label into phenolic products L-phenylpyruvic acid and/or L-phenylacetic acid in addition to *t*-cinnamic acid (Fig. 1). This finding lent further support to possibility 3(b) above. The measurement of both *t*-cinnamic acid and L-phenylpyruvic acid together, by standard spectrophotometric and isotopic methods was possible.

This finding was further supported by observing PAL activities to be hundred times higher than would account for amounts of phenylpropanoid (phaseollin) production in bean tissues exhibiting hypersensitive necrosis on infection with *Colletotrichum lindemuthianum* L. This lack of correlation between PAL activity and amounts of phenylpropanoid accumulated was also reported by Margna [8] using the above assays on several plant systems. He also documented an extensive list of experimental systems in which PAL activities determined by similar methods were too high to explain the amounts of phenylpropanoids that had accumulated. These results could be explained if we were measuring more than one enzyme or more than one product. If we were measuring both *t*-cinnamic acid and L-phenylpyruvic acid together, as described above, we were possibly measuring both PAL and the enzyme responsible for the conversion of L-phenylalanine to L-phenylpyruvic acid (PAT) together.

The similarities in pH and temperature optima of both PAT and PAL, presence of endogenous keto acids, pyridoxal phosphate associated with PAT protein, widespread occurrence of PAT [1, 9] and failure to inhibit both the spectrophotometric and uniformly labelled isotopic assays with D-phenylalanine suggested the possible presence of PAT in homogenates used to assay PAL activity.

*The* L-[1-$^{14}$C]*phenylalanine isotopic assay*

Using 1-C-labelled L-phenylalanine as substrate for PAL activity followed by the separation of labelled *t*-cinnamic acid (a measure of PAL activity) from L-phenylpyruvic acid (a measure of PAT activity) (see Experimental), the ratio of PAL to PAT activity was found to be 1:9. Reports of up to 50% incorporation of L-phenylalanine into L-phenylpyruvic acid is known for different plant systems [10, 11]. Similar results were obtained in tissues known to contain a higher amount of phenylpropanoids and PAL activity. These were bean and potato tuber tissues exposed to continuous light for 24 hr and bean hypocotyl and leaf tissues infected with *Colletotrichum lindemuthianum*.

The low incorporation of L-phenylalanine into *t*-cinnamic acid could be due to (a) loss of measurable *t*-cinnamic acid and/or (b) low amounts of *t*-cinnamic acid produced. Possibility (a) could result from *t*-cinnamic acid being bound to protein. However, the incubation of different amounts of *t*-[$^{14}$C]cinnamic acid in presence of varying concentrations of protein did not result in loss of measurable *t*-cinnamic acid. Possibility (b) could result from presence of an endogenous inhibitor or enzyme competing for the same substrate as PAL. The lack of inhibition of PAL in the various green and non-green systems by varying concentrations of homogenates from other green and non-green systems, suggested the absence of an inhibitor of PAL activity.

From results described above, possibility (iiib) could be due to another enzyme, PAT, competing for the same substrate as PAL, responsible for the production of L-phenylpyruvic acid. Additional evidence for the presence of two enzymes PAT and PAL competing for the same substrate L-phenylalanine came from the use of the Woolf plot (for two enzymes competing for the same substrate [12]) to determine the apparent $K_m$ and individual $V_{max}$ of both PAT and PAL. Using the [1-$^{14}$C]-isotopic assay for PAL activity, PAL was found to have a $k_m$ of 20 mM and $V_{max}$ of 250 pkat. PAT was found to have a higher affinity for L-phenylalanine ($K_m$ 0.03 mM) and lower $V_{max}$ (16.6 pkat), as compared to PAL. These results indicated that low activities of PAL were due to low levels of *t*-cinnamic acid production as phenylalanine was converted largely through the PAT pathway. Under these conditions, a high concentration of L-phenylalanine would be necessary for PAL to reach half maximal velocity. This may explain the reports on negative cooperativity of PAL in several systems, and the conversion of negative to positive cooperativity on purification of PAL [13, 14].

The presence of PAT was experimentally tested for. Using beta-mercaptoethanol to inhibit the incorporation of L-phenylalanine into *t*-cinnamic acid (unpublished data, a measure of PAL activity), PAT, a reversible enzyme, could be made to reduce .2.5 mM NAD in the presence of 0.1 units of alpha-ketoglutarate dehydrogenase, 20–80 mM L-phenylpyruvic acid, 0.2 mM thiamine pyrophosphate, 1.0 mM $MgCl_2$, 2.6 mM coenzyme A and 2.6 mM cysteine. The reader is referred to Brown and Perham [15] for experimental details. This assay for PAT was linear with time and protein concentration in presence of exogenous L-glutamic acid.

*The* L-[1-$^{14}$C]*phenylalanine isotopic assay in absence of measurable PAT activity*

Using L-aspartic acid (in equimolar amounts to L-phenylalanine) to inhibit the incorporation of L-[1-$^{14}$C]phenylalanine into L-phenylpyruvic acid [16] and hence, PAT activity, the incorporation of label into *t*-cinnamic acid and L-phenylpyruvic acid was measured. No labelled L-phenylpyruvic acid was detected by methods described above. Further, no labelled carbon dioxide (trapped as $KHCO_3$) was produced on decarboxylation of keto-acids such as phenylpyruvic acid by hydrogen peroxide. This assay for PAL activity was linear with time and protein concentration in presence of exogenous alpha-ketoglutaric acid for up to two hr. For details of methods used see the Experimental. The absence of an amino acid oxidase was indicated by the necessity of both L-aspartic acid and alpha-ketoglutaric acid for reliable estimation of PAL activities by this method. No measurable PAL activities were obtained with either of these compounds alone or with 20–60 mM L-glutamic acid.

In the presence of L-aspartic acid, PAL was found to have a higher affinity for L-phenylalanine ($K_m$ 1.25 mM) than that obtained when PAT was active. Further, in the

presence of L-aspartic acid, addition of equimolar amounts of D-phenylalanine and L-phenylalanine resulted in 56% inhibition of measurable PAL activity. These results illustrated an inhibition of PAT activities in presence of L-aspartic acid.

*The regulation of phenylpropanoid biosynthesis*

PAL is the first committed enzyme in the pathway leading to phenylpropanoid biosynthesis in higher plants [13]. The flux of L-phenylalanine through the phenylpropanoid pathway is therefore thought to be regulated by PAL [13, 14]. The correlative increase in PAL activity concomitant with increase in phenylpropanoid production [14] and the low catalytic activity (non-equilibrium condition) of PAL as opposed to PAT in conditions optimal for both PAL and PAT activity, favour such a view. In our attempts to understand the metabolic control of PAL activity, it was necessary to use a crude system, because the properties of the enzyme that are relevant to metabolic control are more likely to be preserved. Using such systems the activity of PAL is thought to be regulated solely by changes in substrate concentration (substrate supply and availability) [8] or by factors other than substrate concentration (feed-back inhibition by *t*-cinnamic acid) [13, 14]. These conclusions are based on indirect evidence [8] and on standard methods (spectrophotometric and uniformly labelled isotopic assays) used to assay PAL activity [13, 14]. However, using the corrected [1-$^{14}$C]-isotopic assay for PAL in the absence of PAT activity, PAL was not inhibited by *t*-cinnamic acid ($K_i > 0.6$ mM) but by L-phenylpyruvic acid ($K_i$ 4.16 $\mu$M). Hanson and Havir [13] reported similar observations. However, *t*-cinnamic acid was found to inhibit PAT activity ($K_i$ 1.2 $\mu$M). These results suggest a feed-forward activation [13], as opposed to feedback inhibition as the control mechanism of PAL activity also supported by (i) observations on stimulated rate of removal of active PAL protein by *t*-cinnamic acid using the spectrophotometric assay [14] and (ii) the equilibrium constant of PAT being 6.8 [16a].

If feedback inhibition does not control PAL activity, feed-forward activation indirectly suggests changes in substrate (L-phenylalanine) concentration to be a possible means of control. If the supply of L-phenylpyruvate is kept constant by the controlling activity of prephenate dehydratase, and if the reaction catalysed by PAT is maintained in equilibrium, then the flux of L-phenylalanine into PAL (hence phenylpropanoid metabolism) would be greatly facilitated if protein synthesis were not functioning optimally. The importance of L-phenylalanine as a structural component of phenylpropanoids, low endogenous concentration of unbound L-phenylalanine in plant tissues (0.1–0.2 $\mu$M/g fr. wt), constant L-phenylalanine pool size during rapid fluctuations in phenylpropanoid synthesis, lack of feed-back inhibition of *t*-RNAs for L-phenylalanine and the inverse correlation between protein synthesis and phenylpropanoid biosynthesis suggested availability of L-phenylalanine to be under direct competition between the increase in PAL activity and by the enzymes of protein synthesis [8]. In light of PAT being the enzyme involved in regulating the flux of L-phenylalanine into protein synthesis and/or that necessary for PAL activity (substrate supply), the results described above may favour such a view.

Experiments involving the induction of phenylpropanoid synthesis (phaseollin) in bean cells exhibiting hypersensitive necrosis with *Colletotrichum lindemuthianum* lend further support to such a finding (Fig. 2). Following an initial phase of increase in PAT activity (in the direction L-phenylalanine → L-phenylpyruvic acid), together with production of newly synthesised PAT protein and inactive PAL protein (unpublished data), there occurs a decrease in protein synthesis concomitant with the induction of PAL activity (together with the activation of newly synthesised inactive PAL protein, unpublished data) and decrease in PAT activity (in the direction L-phenylalanine → L-phenylpyruvic acid). In presence of 10 mM exogenous L-phenylalanine uninfected host cells show no phenylpropanoid (phaseollin) production. Such cells show no change in protein synthesis, nor increase in PAL activity. However, such cells show an increase in newly synthesised inactive PAL protein (unpublished data) concomitant with a 15% increase in PAT activity (in the direction L-phenylalanine → L-phenylpyruvic acid). These results illustrate the necessity of both substrate supply and availability for PAL activity and phenylpropanoid biosynthesis. The inverse correlation between PAL activity and protein synthesis is well documented [8]. Results described above using the corrected isotopic assay in absence of PAT activity also suggest that the induced production of L-

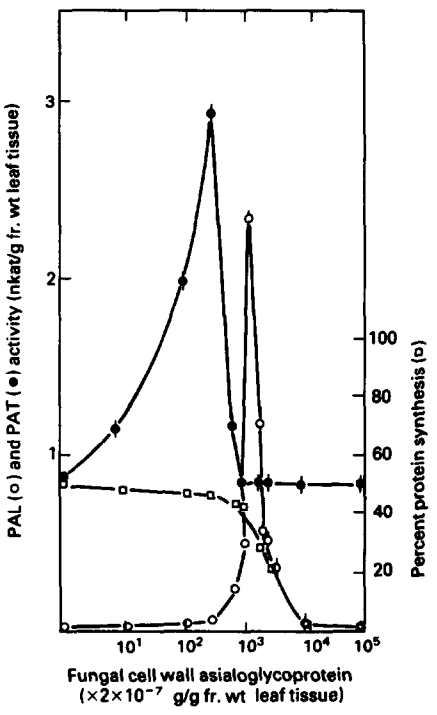


Fig. 2. The activities of PAL (formation of *t*-cinnamic acid) and PAT (formation of L-phenylpyruvic acid) in relation to protein synthesis during hypersensitive necrosis. Leaf cells of the host plant (*Phaseolus vulgaris*) were treated with fungal pathogen (*Collectotrichum lindemuthianum*) cell wall asialoglycoprotein for a period of 10 hrs. Results are interpreted as leaf tissue for convenience. Results shown are the mean of seven different experiments. The bars indicate the maximum and minimum values in an experiment.

phenylpyruvic acid (observed as an apparent increase in PAL activity using standard spectrophotometric methods) preceding the increase in PAL activity may account for the subsequent inhibited production of newly synthesized PAL protein in presence of *t*-cinnamic acid [14].

In conclusion, results using the standard method of assaying PAL activities have yielded considerable variation in the properties attributed to PAL. Amongst these are variations in (i) rates of measurable enzyme activity (as assessed by continuous and stopped assays), (ii) cooperativity (negative to positive cooperativity on purification), (iii) affinities for L-phenylalanine, (4) degrees of inhibition by phenolics, (iv) the temporal and spatial association between PAL and phenylpropanoids, (v) inhibition by compounds such as beta-mercaptoethanol, (vi) presence of isoenzymic forms of PAL protein and (vii) the biphasic response of PAL activity during hypersensitive necrosis and phaseollin production in *Phaseolus vulgaris* var. Prince tissues in response to the pathogen *Colletotrichum lindemuthianum* isolate CMI 112166 [8, 13, 14, 17–19]. These results were not obtained using the corrected assay for PAL, in absence of PAT activity but were obtained in the presence of PAL containing PAT activity. The near charge properties of PAT and PAL, epitopes common to both PAT and PAL protein (unpublished data), and the possibility of a common intermediate (Schiff's base) in the reactions of both PAT and PAL [9, 13] may explain discrepancies regarding the presence of isoenzymic forms of PAL. These results may also help us understand structural aspects involved in the regulation of PAL by L-phenylalanine supply from the PAT protein. The similarities in absorption spectra between *p*-coumaric acid, L-phenylpyruvic acid and *t*-cinnamic acid [in 0.1 M sodium borate buffer, pH 8.8, *p*-coumaric acid was found to have a molar absorption coefficient $\lambda_{max}^{Na_2B_4O_7}$ nm (log ε): 290 (1.4), 265 (1.3)], tyrosine being an alternative substrate for PAT activity, and the involvement of tyrosine in protein synthesis, suggest tyrosine ammonia–lyase activity (TAL, E.C. 4.3.1.5) to be regulated by the same mechanism as PAL.

## EXPERIMENTAL

*Preparation of plant and pathogen material.* Growth of plant material (*Phaseolus vulgaris* L. var. Prince and *Pisum sativum*), rapid isolation of bean leaf cells, hypocotyl-derived bean suspension cultures and callus cultures is described [20]. Seeds of *Zea mays* were grown under identical conditions, but maintained in autoclaved graded horticultural perlite. Discs of *Solanum tuberosum* L. var. Maris Piper were illuminated for 16 hr under white fluorescent light (3300 to 4400 lx). Hypocotyls were excised from 7-day old seedlings. They were used whole or dissected longitudinally with a sterile razor blade and placed cut side upper most on moist filter paper in petri dishes. Sterile distilled $H_2O$ was applied to the cut surfaces and segments illuminated for several hours under white fluorescent light. The pathogen, *Colletotrichum lindemuthianum* isolate CMI 112166 was grown as described [21].

*Induction of phenylpropanoid (phaseollin) production.* The inoculum for whole plant material consisted of 10-day-old mycelium suspended in distilled $H_2O$ at a concn. of 1 mg per ml. Plants were treated by infiltrating leaves and hypocotyls with a Hagborg device. Plants were incubated at 100% relative humidity to stimulate hyphal growth. Control inoculations were carried out with distilled $H_2O$. The number of lesions per g fr. wt leaf material was used to quantify hypersensitive necrosis. Phaseollin was estimated by homogenising leaves in 80% EtOH at a concentration of 1.5 ml per g fr. wt leaf material. The extract was filtered, dried *in vacuo* at 40° and the residue dissolved in distilled $H_2O$, partitioned × 4 against petrol (bp 40–60°). The ether fractions were dried *in vacuo* at 25°, the residue dissolved in 90% EtOH and samples run on silica gel UV 254 TLC plates in $CHCl_3$–MeOH (50:2). Phaseollin ($R_f$ 0.71–0.75) was quantified [22].

The inoculum for rapidly isolated single cells consisted of asialoglycoprotein isolated from 10-day-old mycelium. Cell walls were extracted in $CHCl_3$–MeOH (1:1) followed by acetone. The cell walls were air-dried, and the asialoglycoprotein was heat-released in distilled water at 121° for 20 min, in an autoclave. The asialoglycoprotein was precipitated with 80% acetone, suspended in 20 mM $Na_2HPO_4$–$NaH_2PO_4$ buffer (pH 7.2), re-precipitated with 1 mg Conconavalin A per 5 mg carbohydrate, resuspended and passed through a DEAE-Sephacel column in presence of 0.2 to 0.5 mM methyl-α-D-mannoside to separate conconavalin A from the asialoglycoprotein.

*Separation of t-cinnamic acid from L-phenylpyruvic acid.* Samples were extracted in twice the vol. of toluene. For concns above 17 nM *t*-cinnamic acid, a second extraction with toluene was necessary. The toluene fractions were evaporated to dryness *in vacuo* at 18–25°, dissolved in 90% EtOH, and run on TLC plates (Polygram Cell 300 DEAE cellulose) in 50 mM $Na_2HPO_4$–$NaH_2PO_4$ buffer (pH 7.0). Samples were located by fluorescence (366 nm), and after spraying with 0.05% chromotropic acid. $R_f$ values obtained for *t*-cinnamic acid, L-phenylpyruvic acid, L-phenylacetic acid and L-phenylalanine were 0.24, 0.34, 0.58 and 0.80 respectively.

*Assay for PAL activity by the L-[1-$^{14}$C]phenylalanine assay.* Homogenates were prepared by grinding tissue in liquid nitrogen. 1/10 (w/v) insoluble PVP was added in 0.1 M $Na_2B_4O_7$ buffer (pH 8.8) containing 5 mM L-ascorbic acid at a concentration of 5 ml per g f. wt leaf tissue. The homogenate was filtered and passed through Sephadex G-25. Aliquots of homogenate were pre-incubated for 30 min with 10 mM L-aspartic acid, 20 mM α-ketoglutaric acid and 20 μm pyridoxal-5'-phosphate prior to the addition of 10 mM L-phenylalanine containing 0.09 nM L-[1-$^{14}$C] phenylalanine at 57 mCi per mmol. The reaction mixture was incubated for a further 2 hr. The estimation of protein synthesis is described [19].

*Acknowledgements*—The author wishes to thank Prof. F. R. Whatley (F.R.S.), Dr V. S. Butt and Dr J. O. D. Coleman for valuable discussions. This work was carried out at the Department of Plant Sciences, University of Oxford and is part of a D.Phil. thesis submitted to the University of Oxford, Oxford. Financial support from the University of Oxford, St. Hilda's College, Radhakrishan Memorial Bequest and Prof. F. R. Whatley are gratefully acknowledged.

## REFERENCES

1. Koukol, J. and Conn, E. E. (1961) *J. Biol. Chem.* **236**, 2692.
2. Lamb, C. J., Merritt, T. K. and Butt, V. S. (1971) *Biochem. Biophys. Acta.* **582**, 196.
3. Hanson, K. R. (1981) *Arch. Biochem. Biophys.* **211**, 564.
4. Lowry, R., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
5. Eze, J. M. O. and Dumbroff, E. B. (1982) *Can. J. Bot.* **60**, 1046.
6. Erez, A. (1973) *Plant Physiol.* **51**, 409.
7. Wilkinson, E. M. (1978) D.Phil. Thesis. University of Oxford, Oxford.

8. Margna, U. (1977) *Phytochemistry* **16**, 419.
9. Wightman, F. and Forest, J. C. (1978) *Phytochemistry* **17**, 1455.
10. Jones, D. H. and Northcote, D. H. (1984) *Arch. Biochem. Biophys.* **235**, 167.
11. Stafford, H. A. and Lewis, L. L. (1977) *Plant Physiol.* **60**, 830.
12. Spears, G., Sneyd, J. G. T. and Loten, E. G. (1971) *Biochem. J.* **125**, 1149.
13. Hanson, K. R. and Havir, E. A. (1981) in *The Biochemistry of Plants* Vol. 7 (Stumpf, P. K. and Conn, E. E., eds) p. 577. Academic Press, New York.
14. Dixon, R. A., Dey, P. M. and Lamb, C. J. (1983) *Adv. Enzymol.* **55**, 1.
15. Brown, J. P. and Perham, R. N. (1980) *Biochem. J.* **155**, 419.
16. Forest, J. C. and Wightman, F. (1972) *Can. J. Biochem.* **51**, 332.
16a. Lehninger, A. L. (1982) *Principles of Biochemistry* p. 394. Worth, New York.
17. Jones, D. H. (1984) *Phytochemistry* **23**, 1349.
18. Faye, L. (1975) Thesis. University of Rouen, France.
19. Smith, D. A. and Banks, S. W. (1986) *Phytochemistry* **25**, 979.
20. da Cunha, A. *Physiol. Plant.* (in press).
21. Mathur, R. S., Barnett, H. L. and Lilly, U. G. (1950) *Phytopathology* **40**, 104.
22. Cruickshank, I. A. M. and Perrin, D. R. (1971) *Phytopath. Z.* **70**, 209.