Pergamon

0031-9422(95)00094-1

Phytochemistry, Vol. 39, No. 4, pp. 751–755, 1995

0031-9422/95 $9.50 + 0.00

EFFECT OF PH ON CO_2-ACTIVATED 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE ACTIVITY FROM APPLE FRUIT

FUSAO MIZUTANI,* JIAN GUO DONG and SHANG FA YANG†

Mann Laboratory, Department of Vegetable Crops, University of California, CA 95616, U.S.A.

(*Received in revised form* 4 *January* 1995)

Key Word Index—*Malus domestica*; Rosaceae; apple fruit; ACC oxidase; pH effect; CO_2 activation; kinetic parameters.

Abstract—1-Aminocyclopropane-1-carboxylate(ACC) oxidase, which converts ACC to ethylene, requires CO_2 as an activator for its activity. Using crude extracts of apple fruit, we examined the effect of pH on CO_2-activated ACC oxidase activity. Since gaseous CO_2 lowered pH of the assay media, the final pH of the media was either checked after the reaction, or the pH was maintained by employing CO_2– bicarbonate buffer. At ambient CO_2, the optimum pH was 7.4; at this pH, maximal activity was attained with 4% atmospheric CO_2 as previously reported. As the concentration of CO_2 was increased, the optimum pH gradually shifted toward acidic side. At 20% CO_2, the optimum pH was around 6.7. Thus, the concentration of CO_2 to elicit maximal enzyme activity varied greatly with pH. Dependence of the enzyme activity on CO_2 concentration at various pHs ranging from 6.0 to 7.5 was investigated. While the affinity of the enzyme for CO_2 decreased as the pH decreased, V_{max} increased as the pH decreased, reaching a peak activity at pH 6.7. These results indicate that CO_2 affects ACC oxidase activity not only by serving directly as an ACC oxidase activator but also by modifying the tissue pH which in turn influences the enzyme activity. Furthermore, some enzyme kinetic parameters for various substrates and CO_2 were determined.

INTRODUCTION

The gaseous plant hormone ethylene regulates many aspects of plant growth and development. The final step of its biosynthesis is catalysed by ACC oxidase that converts ACC into ethylene [1]. Because ACC oxidase activity was completely lost upon homogenization of tissues, the enzyme has long been considered as a membrane-bound enzyme that requires membrane integrity for its activity. However, recently ACC oxidase was extracted as a soluble form first from melon fruit, and Fe^{2+} and ascorbate were found to be essential factors for its activity [2]. Subsequently the enzyme was purified and characterized [3–6]. Using plant tissue, carbon dioxide has been shown previously to stimulate the conversion of ACC to ethylene *in vivo* [7–10]. Recently it was also demonstrated that carbon dioxide is an essential activator for extracted ACC oxidase [3, 6, 11]. The optimum pH of ACC oxidase was determined as about 7.5 with melon fruit [12], 7.5–8.0 with avocado fruit [13], and 7.2 and 7.6 with apple fruit [14]. In these cases, the optimum pH was assayed under ambient CO_2 conditions. Dong *et al.* [3] reported that the *in vitro* enzyme activity analysed at pH 7.2 increased with increasing CO_2 concentrations to a maximum at 4%. Fernández *et al.* [15] identified CO_2 rather than HCO_3^- as the active species involved in the activation process. CO_2 passes very rapidly through membranes [16] and lowers intracellular pH owing to its hydration and dissociation into H^+ and HCO_3^- [17]. In this communication we report the effect of pH on the kinetics of CO_2-activated ACC oxidase activity *in vitro* and describe the influence of CO_2 upon some kinetic parameters of the enzyme.

RESULTS AND DISCUSSION

Enzyme assay

In the homogenate of apple fruits, it has been shown that ACC oxidase activity is associated with the pellet fraction and can be solubilized with Triton X-100 or PVPP [5]. In this work, we observed that, when the crude extract was assayed in the presence of Triton X-100, the activity increased two-fold as compared with that in the absence of Triton X-100 (data not shown). Furthermore, no difference was found in the enzyme activity between the crude extract and the supernatant after centrifugation at 28 000 *g* for 20 min following the addition of 0.1%

*College of Agriculture, Ehime University, Tarumi Matsuyama 790, Japan.

†Present address: Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong.

Triton X-100. This indicates that ACC oxidase was solubilized in the assay system supplemented with 0.1% Triton X-100. So we used the crude extract supplemented with 0.1% Triton X-100 as the enzyme source without further purification. Within 1 hr of assay, the activity was nearly linear but thereafter declined. Hence we routinely assayed the enzyme activity after incubating for 30 min.

Since HCN and dehydroascorbate are reaction products of the enzyme reaction [3], we examined whether end product inhibition is involved in the decline of the activity. At 1 mM, KCN exerted 47% inhibition and dehydroascorbate 11% inhibition, but no synergistic effect was observed when the two compounds were combined (data not shown). Since HCN is effectively metabolized into β-cyanoalanine in the tissue, such an inhibition is not expected to be significant in intact plant tissues [18].

Effect of pH and CO_2 on enzyme activity

The enzyme activity when KH_2PO_4–NaOH buffer (pH 5.8–8.0) and Tris–HCl buffer (pH 7.0–9.0) were used as assay media in combination with different CO_2 concentrations is shown in Fig. 1. Increasing CO_2 concentrations in the assay system resulted in a drastic decrease in pH of the medium. For example, at 20% CO_2 the initial pH of 7.0 and 8.0 in the phosphate buffer dropped to around 6.5–6.7, respectively. Under ambient CO_2 conditions, the optimum pH for the enzyme activity was 7.4. However, as the CO_2 concentration increased, the optimum pH gradually decreased and the maximum activity increased. The optimum pH and the maximum activity at various fixed CO_2 concentrations in the phosphate buffer were 7.0 and 190 nl g^{-1} hr^{-1} at 1% CO_2, 6.9 and 270 nl g^{-1} hr^{-1} at 4% CO_2, and 6.7 and 350 nl g^{-1} hr^{-1} at 20% CO_2, respectively (Fig. 1). A similar pattern was obtained when 0.1 M Tris–HCl buffer (pH 7.0–9.0), a more alkaline buffer, was employed.

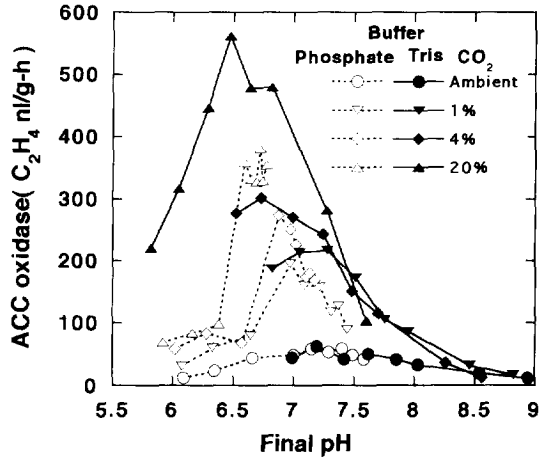

Fig. 1. Effect of pH and CO_2 on ACC oxidase activity. Enzyme activities were assayed in a 0.1 M KH_2PO_4–NaOH buffer (pH 5.8–8.0) or a 0.1 M Tris–HCl buffer (pH 7.0–9.0) with ambient, 1, 4 and 20% CO_2 concentrations. After the enzyme assay, the final pH of the reaction mixture was determined.

Furthermore, we used an appropriate concentration of bicarbonate at a given CO_2 concentration and a given pH according to the Henderson–Hasselbach equation, so that the pH of the assay medium is stabilized in the presence of CO_2 [19]. Again, increasing CO_2 concentrations caused not only a shift of the optimum pH to the acidic side but also increased the maximum enzyme activity (Fig. 2A). The effect of varied CO_2 concentrations at different fixed pHs on ACC oxidase activity is shown in Fig. 2B. At pH 7.5, the activity increased to a maximum at 4% CO_2, but declined with higher CO_2 concentrations.

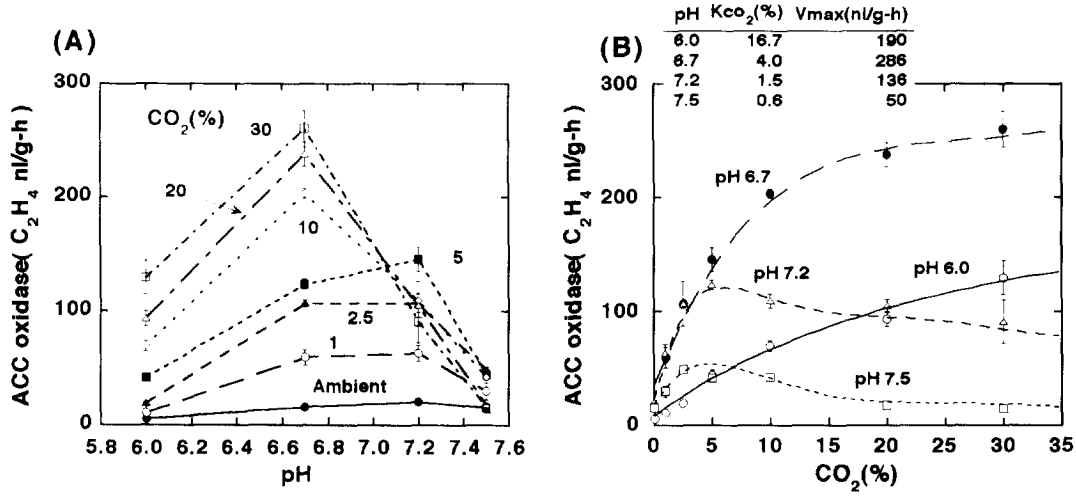

Fig. 2. Effect of pH and CO_2 on ACC oxidase activity. The reaction was carried out as described in Experimental, employing 0.2 M MES buffer at pH 6.0 or 0.2 M MOPS buffer at pH 6.7, 7.2 and 7.5. Various CO_2 concentrations were employed as indicated, in combination with appropriate amounts of bicarbonate so that the pHs of the reaction mixtures are maintained. (A) ACC oxidase activity versus pH at different fixed CO_2 concentrations; (B) ACC oxidase activity versus CO_2 concentrations at four different pHs.

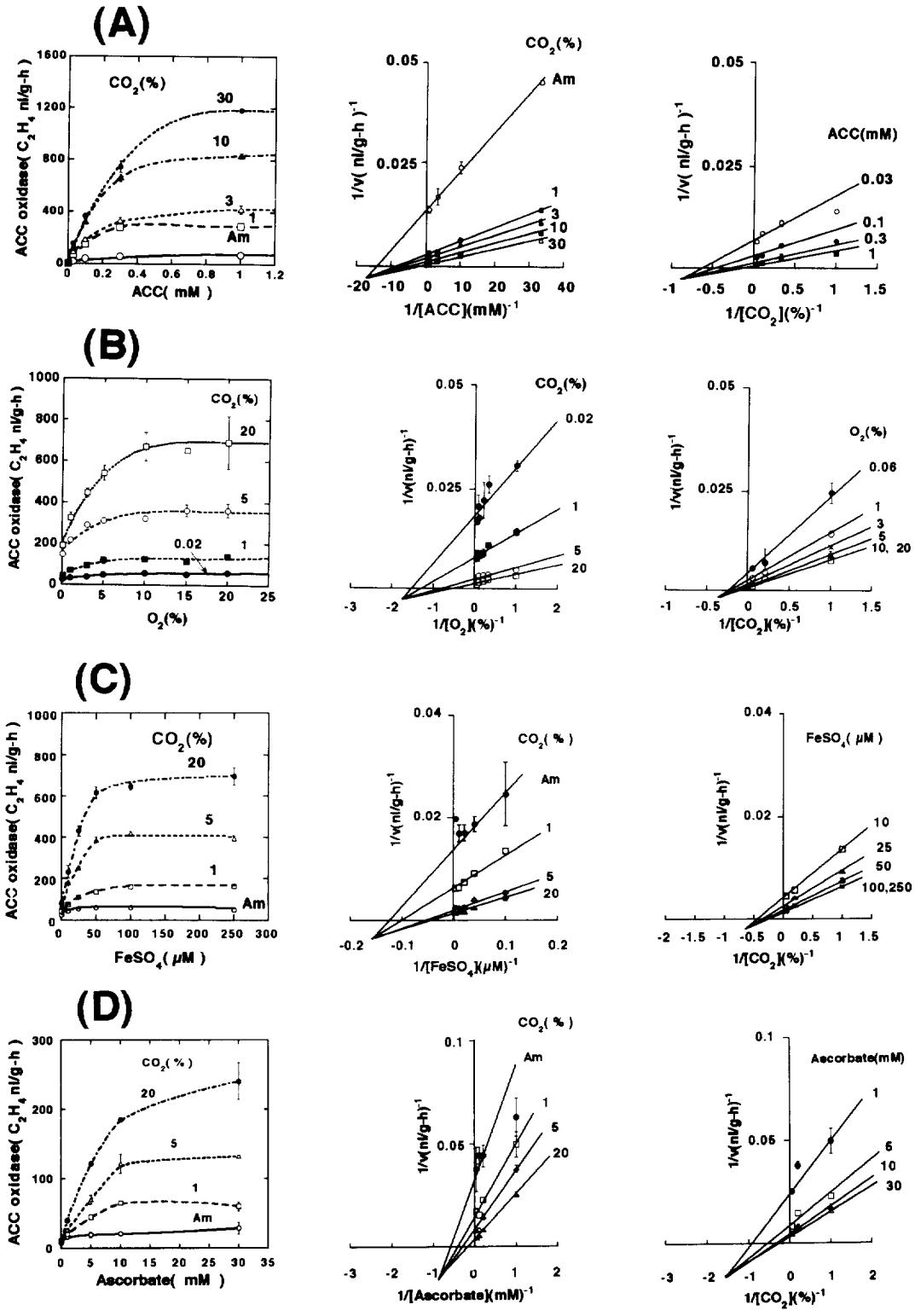

Fig. 3. The v vs $[S]$ plots in the presence of different concentrations of CO_2 and their Lineweaver–Burk plots ($1/v$ vs $1/[S]$ and $1/v$ vs $1/[CO_2]$). Different concentrations of CO_2–bicarbonate mixtures with 0.2 M MOPS buffer (pH 6.7) were used as the assay medium.

On the other hand, at pH 6.0, the activity increased with increasing CO_2 concentrations and did not level off, even at 30% CO_2. The apparent K_m for CO_2 was 0.6% at pH 7.5, but increased to 17% as pH was lowered to 6.0. However, the V_{max} was the greatest at pH 6.7. It was recently reported that the pH optimum of the *in vitro* ACC oxidase from sunflower root tissue was shifted lower with higher CO_2 concentrations [20].

Using a highly purified ACC oxidase from apple fruit, it was previously reported that at an assay pH of 7.2 the enzyme activity increased with increasing CO_2 concentrations to a maximum at 4% and 0.5% CO_2 gave a half-maximal activity [3]. These values are in good agreement with our present data.

The *in vivo* ACC oxidase activity in tobacco leaf discs showed two saturation curves at 2 and 10% CO_2 [21]. Such an observation can be explained by our present data. Namely, CO_2 has two functions; one is to lower pH of the tissues and the other is to activate ACC oxidase. The saturation concentration of CO_2 is low at higher pHs, but as the pH becomes lower, the saturation concentration of CO_2 progressively increases (Fig. 2). Thus, in the presence of low concentrations of CO_2, the cellular pH of the tissues is little affected by the CO_2 so that the saturation concentration of CO_2 is low. However, as the CO_2 concentration is increased, the cellular pH of the tissues decreases, and this results in a higher saturation concentration of CO_2.

Therefore unless otherwise noted, we employed 20% CO_2 supplemented with 105 mM HCO_3^- at pH 6.7 buffered with 0.2 M MOPS for the enzyme assay in the subsequent experiments.

Enzyme kinetics

It has been established that ACC oxidase utilizes ACC, O_2, and ascorbate as the substrates, and CO_2 and Fe^{2+} as activators [3]. We have, therefore, examined some kinetic parameters of ACC oxidase activation by CO_2 in relation to other factors (Fig. 3). Increasing CO_2 concentrations stimulated the V_{max} of ACC oxidase and also increased the apparent K_m of the enzyme with respect to ACC, O_2, Fe^{2+} and ascorbate as revealed by Lineweaver–Burk plots (Fig. 3). Reciprocally increasing concentrations of these factors resulted in the increase in K_m values for CO_2. Using purified ACC oxidase, Fernández *et al.* [15] recently found that the apparent K_m for ACC and O_2 increased with increasing CO_2 concentrations. Smith and John [6] also reported that HCO_3^-/CO_2 increased the apparent K_m towards ACC and O_2.

In summary, our present data indicate that CO_2 can affect ACC oxidase *in vivo* in two ways: (a) it activates ACC oxidase by directly interacting with the enzyme protein, and (b) it lowers the tissue pH which results in lowered affinity of the enzyme toward CO_2, but increased the V_{max} at higher CO_2 concentrations. The optimal pH of the enzyme reaction has been determined in this study to be 6.7, at which the concentration of CO_2 giving half-maximal activity is 4.0%. In order to obtain maximal and comparable activity reported by various researchers, we suggest that ACC oxidase be assayed under 20% atmosphere CO_2 at pH 6.7

EXPERIMENTAL

Plant material. Apple (*Malus domestica* Borkh. cv Golden Delicious) fruit at climacteric stage as confirmed by C_2H_4 production rate were used as the source of ACC oxidase. For each experiment, the cortical tissue of the same fruit was employed.

Enzyme extraction and assay. Cortical tissue was ground with a pestle and mortar in 2 ml g^{-1} tissue of extraction medium containing 0.1 M Tris–HCl (pH 7.4), 10%(v/v) glycerol and 30 mM Na ascorbate. However, ascorbate was excluded from the extraction medium in the enzyme kinetic experiments using different concns of ascorbate; in this case a slight reduction in the enzyme activity was observed. Following the addition of 0.1% Triton X-100, the homogenate was filtered through 4 layers of cheesecloth. Since there was no difference in the enzyme activity between the filtrate and the supernatant after centrifugation at 28000 *g* at 1° for 20 min, the filtrate was used as the enzyme source in this work. All procedures were carried out at 4°.

The enzyme activity was assayed in a sealed 15-ml test tube, which contained, in a total vol of 1.56 ml, 0.1 M KH_2PO_4–NaOH buffer (pH 5.8, 6.3, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0) or 0.1 M Tris–HCl buffer (pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.5 and 9.0), 10% glycerol, 30 mM Na ascorbate, 50 μM $FeSO_4$, 1 mM ACC, 0.1% Triton X-100, various amounts of gaseous CO_2 and 0.2 ml crude extract. The same amount of air was withdrawn from the test tubes just before CO_2 injection. The test tubes were shaken and stood for 10 min for equilibration between atmospheric and dissolved CO_2. After the final addition of crude extract and rapid shaking, the test tubes were transferred to a reciprocating shaker (90 cycles min^{-1}) at 30°. C_2H_4 produced in the head space of the tube after 30 min incubation was determined by GC. Since gaseous CO_2 lowers the medium pH, the final pH of the medium was checked with a pH meter immediately after C_2H_4 determination.

In other experiments, 0.2 M MES (pH 6.0) and 0.2 M MOPS (pH 6.5–7.5) buffers were employed as the assay buffers. In order to stabilize the pH of the assay medium due to the presence of gaseous CO_2, calculated amounts of $NaHCO_3$ were added in combination with 1–30% gaseous CO_2 according to the method described in ref. [19]. The pH of the medium was checked after C_2H_4 determination.

Enzyme kinetics. In this study we used 12-ml plastic syringes capped with rubber septums instead of test tubes because the syringe plunger is movable so that we can easily adjust the concn of O_2 and CO_2 in the head space of the reaction syringes. After filling the reaction syringe with a given amount of N_2, a desired amount of O_2 or CO_2 in a syringe was injected with a hypodermic needle through the septum of the reaction syringe by simultaneously moving both plungers. We employed bicar-

6.7 as the assay medium, which was supplemented with 10% (v/v) glycerol, and various amounts of substrates and co-factors as specified above. O_2 and CO_2 concns in reaction syringes were checked by withdrawing 0.5 ml gas samples which were analysed separately with an O_2 analyser and an IR gas analyser immediately after C_2H_4 determination.

Acknowledgements— This work was supported by a research grant (MCB-9303801) from the U.S. National Science Foundation. This work was conducted while F.M. held a travel grant from the Ministry of Education of Japan.

REFERENCES

1. Yang, S. F. and Hoffman, N. E.(1984) *Ann. Rev. Plant Physiol.* **35**, 155.
2. Ververidis, P. and John, P. (1991) *Phytochemistry* **30**, 725.
3. Dong, J. G., Fernández-Maculet, J. C. and Yang, S. F. (1992) *Proc. Natl Acad. Sci. U.S.A.* **89**, 9789.
4. Dupille, E., Rombaldi, C., Lelièvre, J. M., Cleyet-Marel, J. C., Pech, J. C. and Latché, A. (1993) *Planta* **190**, 65.
5. Fernández-Maculet, J. C. and Yang, S. F. (1992) *Plant Physiol.* **99**, 751.
6. Smith, J. J. and John, P. (1993) *Phytochemistry* **32**, 1381.
7. Dhawan, K. R., Bassi, P. K. and Spencer, M. S. (1981) *Plant Physiol.* **68**, 831.
8. Kao, C. H. and Yang, S. F. (1982) *Planta* **155**, 261.
9. McRae, D. G., Coker, J. A., Legge, R. L. and Thompson, J. E. (1983) *Plant Physiol.* **73**, 784.
10. Cheverry, J. L., Sy, M. O., Pouliquen, J. and Marcellin, P. (1988) *Physiol. Plant.* **72**, 35.
11. Dilley, D. R., Kuai, J., Poneleit, L., Zhu, Y., Pekker, Y., Wilson, I. D., Burmeister, D. M., Gran, C. and Bowers, A. (1992) in *Cellular and Molecular Aspects of the Plant Hormone Ethylene* (Pech, J. C., Latché, A. and Balagué, C., eds), p. 46. Kluwer Academic Press, Dordrecht.
12. Smith, J. J., Ververidis, P. and John, P. (1992) *Phytochemistry* **31**, 1485.
13. McGarvey, D. J. and Christoffersen, R. E. (1992) *J. Biol. Chem.* **267**, 5964.
14. Kuai, J. and Dilley, D. R. (1992) *Postharvest Biol. Technol.* **1**, 203.
15. Fernández-Maculet, J. C., Dong, J. G. and Yang, S. F. (1993) *Biochem. Biophy. Res. Commun.* **193**, 1168.
16. Simon, S. A. and Gutknecht, J. (1980) *Biochim. Biophy. Acta* **596**, 352.
17. Kotyk, A. (1989) in *Intracellular pH and Its Measurement* (Kotyk, A. and Slavík, J., eds), p. 15. CRC Press, Boca Raton, Florida.
18. Yip, W. K. and Yang, S. F. (1988) *Plant Physiol.* **88**, 473.
19. Umbreit, W. W. (1957) in *Manometric Techniques—A Manual Describing Methods Applicable to the Study of Tissue Metabolism* (Umbreit, W. W., Burris, R. H. and Stauffer, J. F., eds), p. 18. Burgess, Minneapolis.
20. Finlayson, S. A. and Reid, D. M. (1994) *Phytochemistry* **35**, 847.
21. Philosoph-Hadas, S., Aharoni, N. and Yang, S. F. (1986) *Plant Physiol.* **82**, 925.