Pergamon

PII: S0031-9422(97)00080-0

*Phytochemistry*, Vol. 45, No. 5, pp. 869–877, 1997

0031-9422/97 $17.00+0.00

# ROOT AND LEAF SPECIFIC ACC OXIDASE ACTIVITY IN CORN AND SUNFLOWER SEEDLINGS

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(*Received in revised form* 5 *December* 1996)

**Key Word Index**—*Helianthus annuus*; Compositae; Sunflower; *Zea mays*; Graminaceae; corn; enzymology; ACC oxidase; $CO_2$; *in vitro* activity; isoenzyme.

**Abstract**—Ethylene production by roots and leaves of sunflower and corn with exposure to varying $CO_2$ concentrations was studied. $CO_2$ strongly promoted ethylene production by the leaves of both species, but did not promote ethylene production by roots; *in vivo* analysis of ACC oxidase activity with and without 5% $CO_2$ showed that $CO_2$ promoted activity in leaves while it had little or no effect on ACC oxidase activity in roots. *In vitro* analysis of ACC oxidase extracted from roots and leaves of the two species revealed that substrate $K_m$s, $K_{CO_2}$ and response to pH were different for enzyme extracted from the two organs. These differences were consistent between the two species, indicating that this may be a general phenomenon. ACC and ascorbate levels measured in the different organs corresponded with the differences observed in the $K_{ACC}$ and $K_{ascorbate}$. The data suggest that ACC oxidase may exist as organ specific isoenzymes which are tailored to the environmental and physiological status of each organ. 

## INTRODUCTION

The biosynthetic pathway for ethylene production in higher plants involves the production of ACC from *S*-adenosylmethionine by ACC synthase, and the subsequent oxidation of ACC to ethylene by the enzyme ACC oxidase [1, 2]. The control of ethylene biosynthesis by higher plants is possible at several points in the biosynthetic pathway. Most research has focused on ACC synthase, as experiments indicate that increased ethylene production is preceded by an increase in ACC production and exogenous ACC is rapidly converted to ethylene [3]. Experiments have shown that treatments elevating ethylene production also increase ACC levels and ACC synthase activity [4–6]. ACC synthase exists as a multigene family in many species; some of these genes appear to encode isoforms which may be differentially expressed during development and in response to various stimuli [7, 8].

The biochemical properties of ACC oxidase have been characterized from various fruits, and also from roots of sunflower [9–11]. ACC oxidase appears to be a soluble monomeric enzyme with a molecular mass of 35 000 to 41 000 Da, requiring three substrates for activity including ACC, dioxygen and ascorbate, and using ferrous iron as a cofactor [9, 10, 12, 13]. ACC oxidase has been cloned from several species [14–16], and apparently exists as a multigene family in tomato plants, where 3 different ACC oxidase genomic clones have been identified [17]; two cDNA clones have been isolated from mung bean hypocotyls, one full length and one partial [18]. Recently differential expression of the three tomato ACC oxidase genes has been demonstrated; these genes exhibit both tissue specificity and stimulus specificity [19]. Tissue specific ACC oxidase clones, including root and leaf specific genes, have also been amplified from sunflower (Liu and Reid, unpublished data).

Early work on the regulation of ethylene biosynthesis showed that leaves increased ethylene production in response to elevated levels of atmospheric $CO_2$, an effect that could be attributed to an increase in ACC oxidase activity as assayed *in vivo* [20, 21]. Later it was shown that $CO_2$ also dramatically stimulated ACC oxidase activity *in vitro* [10, 22]. We have previously shown that as for all other ACC oxidases tested, ACC oxidase activity from sunflower roots is strongly stimulated by high $CO_2$ levels when assayed *in vitro* [11]. However, we find that exogenous $CO_2$ does not stimulate ethylene production by the roots *in vivo* [23]. Since it is well established that leaves of sunflower are capable of responding to $CO_2$ *in vivo* by increasing ethylene production, we sought to test

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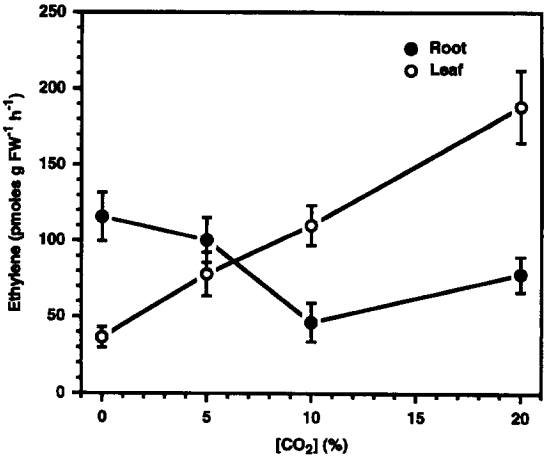


Fig. 1. The effect of $CO_2$ on the rate of ethylene production by excised roots and leaves of corn seedlings after 20 min incubation in the light. $n = 5$, mean ± standard error.

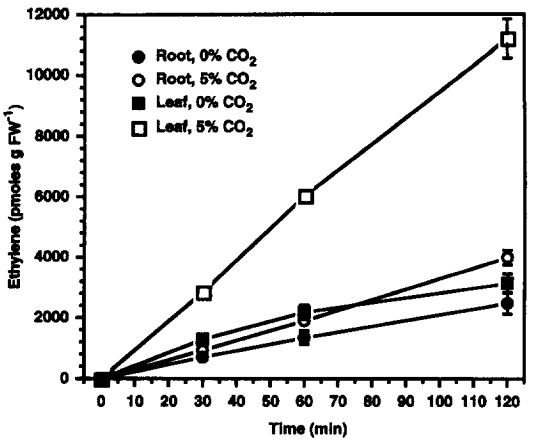


Fig. 2. Ethylene evolution with time from excised roots and leaves of corn seedlings, incubated with 0 or 5% $CO_2$ in the light, with 1 hr 5 mM ACC pre-treatment (a measure of *in vivo* ACC oxidase activity). $n = 5$, mean ± standard error.

whether ACC oxidase activity from roots was different from that in leaves. Additionally we wanted to test whether any phenomenon observed would be general or specific, so corn was selected as an additional species, due to its relatively wide evolutionary divergence from sunflower.

The purpose of this study was to examine the effects of $CO_2$ on *in vivo* ethylene production of roots and leaves of sunflower (*Helianthus annuus* L. var. Dahlgren 131) and corn (*Zea mays* L. cv. Texas 5855), and to characterize ACC oxidase activity from the same organs. Additionally we were interested in measuring ACC and ascorbate levels in these organs in an effort to reconcile substrate levels with the kinetic results obtained.

## RESULTS

### *The effect of* $CO_2$ *on ethylene production by excised roots and leaves*

The effect of $CO_2$ on ethylene production by corn is shown in Fig. 1. Ethylene production by roots decreased as $CO_2$ increased to 20%, whereas ethylene production by leaves steadily increased with increasing $CO_2$. At 20% $CO_2$ leaves produced about 5 times as much ethylene as they did at 0%. Sunflower showed the same response as corn to elevated $CO_2$. While an attempt was made to scavenge all $CO_2$ from the tube using KOH as a trap, it is probable that the concentration of $CO_2$ within the tissues was higher than 0%, due to respiration.

### *The effect of* $CO_2$ *on ACC oxidase activity of roots and leaves measured* in vivo

*In vivo* ACC oxidase activity of corn, at 0% and 5% $CO_2$ is illustrated in Fig. 2. Ethylene production was linear for the 2 hr duration for all the assays, except in leaves at 0% $CO_2$. Corn roots showed only a slight stimulation of ACC oxidase activity when the $CO_2$ concentration was increased from 0 to 5%; however, the increase in ACC oxidase activity with $CO_2$ was much greater in the case of leaves. Sunflower showed a similar response, except that ACC oxidase activity in roots was not stimulated at all by $CO_2$. When *in vivo* ACC oxidase activity of leaves was assayed in darkness, $CO_2$ again stimulated ethylene production, but to a lesser extent than in the light (data not shown). The response in sunflower leaves was similar to that in corn leaves (data not shown).

### In vitro *ACC oxidase from roots and leaves*

Figure 3 represents the response of corn ACC oxidase to pH. Enzyme from both organs showed a narrow range of activity, and a pH optimum near 6.8. Above and below the optimum, the responses of root and leaf were significantly different. At lower pH the root enzyme showed greater inhibition than the leaf, while at higher pH the leaf enzyme showed a more rapid loss of activity. The response of sunflower root and leaf ACC oxidase to pH was similar to that of corn (Table 1).

*In vitro* corn ACC oxidase activity with $CO_2$ is illustrated in Fig. 4. The activity of extracted ACC oxidase from both leaves and roots was stimulated dramatically by $CO_2$. Eadie–Hofstee plots gave a $K_{CO_2}$ of 12.8% for the root enzyme, and 2.93% for the leaf enzyme. Sunflower gave similar results with a root $K_{CO_2}$ 2.3 times higher than the leaf $K_{CO_2}$ (Table 1).

The response of corn ACC oxidase to $O_2$ showed typical hyperbolic kinetics as can be seen in Fig. 5. The $K_m$ for $O_2$ was about four times higher in the leaf than the root enzyme (2.68% leaf, vs. 0.66% root). The root enzyme always showed a disproportionately large amount of activity at the very lowest concentration. This activity could not be explained by the amount of oxygen measured in these tubes (the residual $O_2$ level present even with rigorous purging

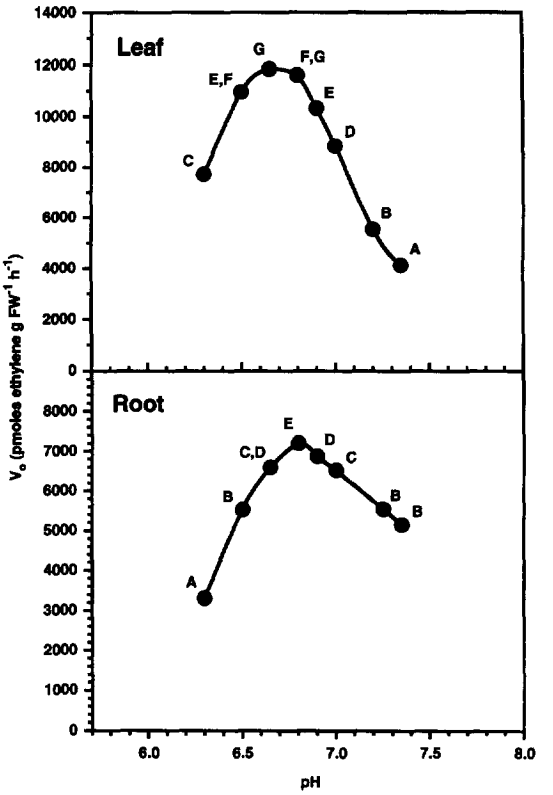


Fig. 3. Dependence of corn root and leaf ACC oxidase activity on pH. $n = 12$. Data points with different letters are significantly different at $\alpha = 0.05$.

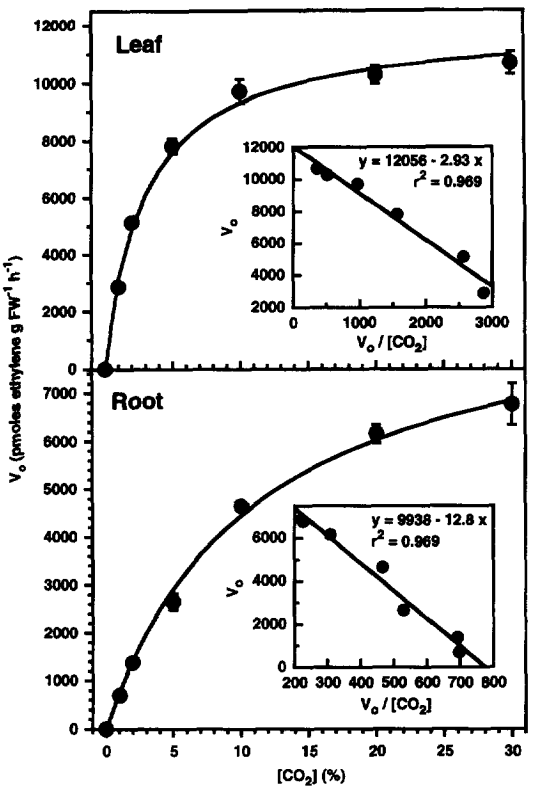


Fig. 4. Dependence of corn root and leaf ACC oxidase activity on $CO_2$ concentration. Insets are Eadie–Hofstee plots. $n = 4$, mean $\pm$ standard error. Ninety-five per cent confidence intervals of the slope of the Eadie–Hofstee regressions do not overlap.

was 0.085%). Similar results were obtained for sunflower (Table 1), and again an unexplained amount of enzyme activity was observed in the root extract at low oxygen levels.

Figure 6 shows the response of corn ACC oxidase to ACC concentration assayed at the standard pH 6.8. In both cases the enzyme was saturated by 2–5 mM ACC. Enzyme activity from both organs showed similar $K_m$s for ACC, with the root giving a slightly higher $K_{ACC}$ of 291 $\mu$M, and the leaf 212 $\mu$M. Identical assays performed at pH 6.5 gave very different results, as can be observed in Fig. 7: at pH 6.5 the leaf $K_m$ was elevated dramatically to 667 $\mu$M, while the $K_{ACC}$ for the root activity did not change appreciably ($K_m = 275$ $\mu$M). At pH 6.5 sunflower leaves gave a $K_m$ for ACC of 331 $\mu$M, and in roots this value was 161 $\mu$M. The $K_{ACC}$ for enzyme activity from both sunflower organs was reduced about 50% when assayed at pH 6.8 (Table 1).

The corn root ACC oxidase had an optimal ascorbate concentration between 10 and 20 mM, with higher concentrations becoming inhibitory to enzyme activity (Fig. 8). The leaf activity exhibited a similar response to ascorbate, with a slightly higher optimum

Table 1. Summary of ACC oxidase properties

| | Sunflower | | | | Corn | | | |
|---|---|---|---|---|---|---|---|---|
| | Root | | Leaf | | Root | | Leaf | |
| Parameter ($x$) | $K_x$/opt | $r^2$ | $K_x$/opt | $r^2$ | $K_x$/opt | $r^2$ | $K_x$/opt | $r^2$ |
| Temperature (°) | 30 | NA | 35 | NA | 35 | NA | 35 | NA |
| pH | 6.8 | NA | 6.8 | NA | 6.8 | NA | 6.7 | NA |
| $CO_2$ (%) | 5.05 | 0.985 | 2.15 | 0.992 | 12.8 | 0.969 | 2.93 | 0.969 |
| $O_2$ (%) | 0.73 | 0.980 | 1.64 | 0.969 | 0.66 | 0.998 | 2.68 | 0.996 |
| ACC ($\mu$M), pH 6.5 | 161 | 0.968 | 331 | 0.991 | 275 | 0.962 | 667 | 0.980 |
| ACC ($\mu$M), pH 6.8 | 91 | 0.979 | 178 | 0.975 | 291 | 0.990 | 212 | 0.985 |
| Ascorbate (mM) | 1.83 | 0.963 | 8.29 | 0.992 | 2.67 | 0.983 | 4.42 | 0.980 |
| Fe ($\mu$M) | 1.28 | 0.930 | 1.11 | 0.930 | 2.63 | 0.985 | 2.77 | 0.963 |

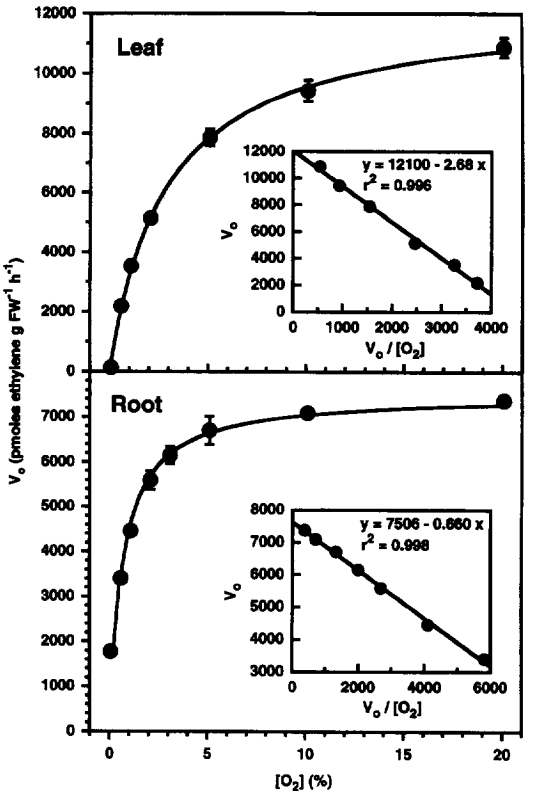


Fig. 5. Dependence of corn root and leaf ACC oxidase activity on $O_2$ concentration. Insets are Eadie–Hofstee plots. $n = 4$, mean ± standard error. Ninety-five per cent confidence intervals of the slope of the Eadie–Hofstee regressions do not overlap.

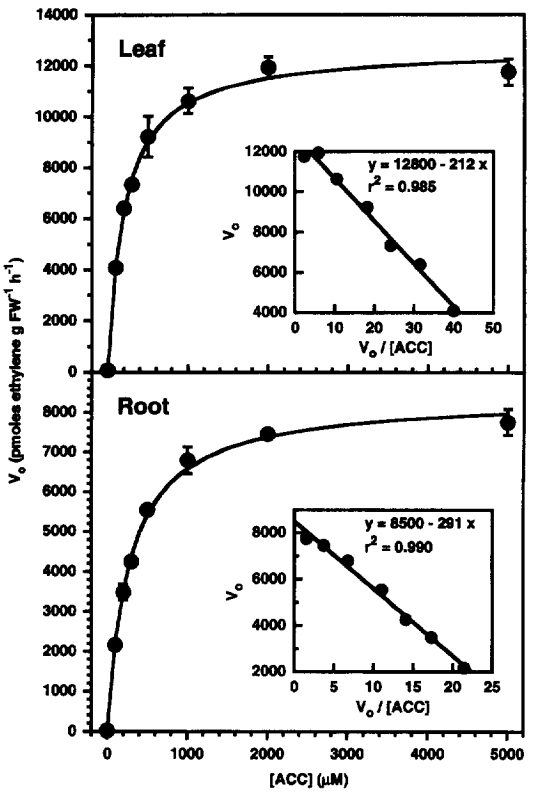


Fig. 6. Dependence of corn root and leaf ACC oxidase activity on ACC concentration at pH 6.8. Insets are Eadie–Hofstee plots. $n = 4$, mean ± standard error. Ninety-five per cent confidence intervals of the slope of the Eadie–Hofstee regressions do not overlap.

level. The $K_m$ for ascorbate for the corn leaf enzyme was calculated to be 4.42 mM, while the corn root gave a value of 2.67 mM. Sunflower showed a similar response, though the differences in $K_m$ were greater (Table 1).

The ACC oxidase response to $FeSO_4$ was not dependent on the extracted organ. ACC oxidase activity extracted from both corn organs gave a $K_{FeSO_4}$ of 2.6 to 2.8 $\mu$M, and activity from both sunflower organs also gave a similar $K_{FeSO_4}$ (Table 1). There were small, non-significant, differences observed in the temperature optima for ACC oxidase from the two sunflower organs. Temperature optima for the ACC oxidase from corn roots and leaves were identical (Table 1).

In all cases the differences observed were reproducible and consistent between repeated extractions, with minor variation.

*ACC and ascorbate oxidized ascorbate levels in roots and leaves*

ACC levels in corn and sunflower leaves and roots are represented in Table 2. Sunflower had slightly higher ACC levels than corn, though the differences were not great. The leaves of both species contained more ACC than the roots, though again the differences were not great.

Table 2 also shows the ascorbate and oxidized ascorbate concentrations in corn and sunflower roots

Table 2. ACC and ascorbate levels in roots and leaves of sunflower and corn. For ACC $n = 3$, mean ± standard error, for ascorbate and oxidized ascorbate $n = 6$, mean ± standard error

| Organ | ACC (nmol g fr. wt$^{-1}$) | Ascorbate ($\mu$mol g fr. wt$^{-1}$) | Oxidized ascorbate ($\mu$mol g fr. wt$^{-1}$) |
|---|---|---|---|
| Sunflower root | 1.63 ± 0.31 | 0.14 ± 0.01 | 0.273 ± 0.02 |
| Sunflower leaf | 2.45 ± 0.47 | 6.72 ± 0.24 | 1.36 ± 0.16 |
| Corn root | 1.30 ± 0.08 | 0.79 ± 0.06 | 0.18 ± 0.02 |
| Corn leaf | 1.76 ± 0.07 | 1.86 ± 0.05 | 0.69 ± 0.02 |

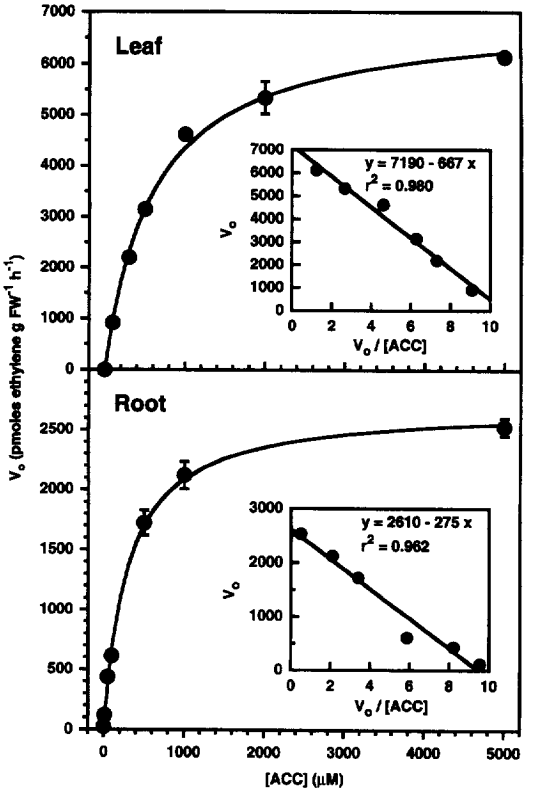


Fig. 7. Dependence of corn root and leaf ACC oxidase activity on ACC concentration at pH 6.5. Insets are Eadie–Hofstee plots. $n = 4$, mean ± standard error. Ninety-five per cent confidence intervals of the slope of the Eadie–Hofstee regressions do not overlap.

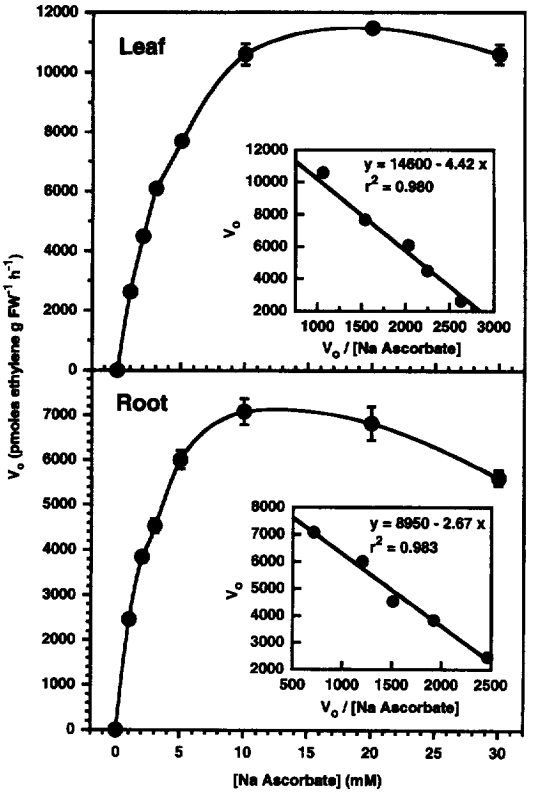


Fig. 8. Dependence of corn root and leaf ACC oxidase activity on ascorbate concentration. Insets are Eadie–Hofstee plots. $n = 4$, mean ± standard error. Ninety-five per cent confidence intervals of the slope of the Eadie–Hofstee regressions do not overlap.

and leaves. Sunflower leaves contained the most ascorbate, with over 30 times as much as sunflower roots. Corn leaves also had more ascorbate than corn roots; however, leaves contained only about twice as much ascorbate as roots. Sunflower roots possessed more oxidized ascorbate than ascorbate, while sunflower leaves, and both corn leaves and roots contained more ascorbate than oxidized ascorbate. Derivatized dehydroascorbate (dehydroascorbate osazone) obtained from plant tissues showed an absorbance spectrum identical to that obtained using pure, commercially obtained chemicals.

## DISCUSSION

Our work initially focused on the response of roots and leaves to environmental $CO_2$ levels. Previous reports showed that $CO_2$ was responsible for stimulating ACC oxidase activity and ethylene production from leaves [20, 21], and subsequent experiments performed *in vitro* corroborated the earlier evidence by showing a dramatic stimulation of ACC oxidase activity when $CO_2$ or bicarbonate was included in the assay [10, 22]. Using sunflower roots, we also found that $CO_2$ stimulated ACC oxidase activity *in vitro* [11]; however, we were not able to increase ethylene production from roots *in vivo* with $CO_2$, nor decrease production by removing $CO_2$ [23]. As shown in this paper, ethylene production in roots of either sunflower or corn was not dependant on atmospheric $CO_2$, while ethylene production by leaves of both species was. By pre-treating seedlings with high levels of ACC we were able to show that $CO_2$ has a great stimulatory effect on *in vivo* ACC oxidase activity in leaves of both corn and sunflower, while it has a minimal effect on *in vivo* ACC oxidase from roots. Using an *in vivo* ACC oxidase analysis with kiwi fruit, Rothan and Nicolas report that high $CO_2$ concentrations depress ethylene production by reducing the efficiency of ACC oxidase [24]. Results obtained previously from sunflower roots indicate that the efficiency of ACC oxidase increases with $CO_2$ in this tissue, as the ratio $V_{max}$ to $K_m$ increases from 26.3 to 76.1 when assayed *in vitro* at ambient and 5% $CO_2$ [11]. It appears that a decrease in ACC oxidase efficiency is not responsible for the lack of a promotive effect of $CO_2$ on ethylene production by roots. More likely the responses observed reflect the internal $CO_2$ concentrations of the tissues. The leaf is a porous structure well suited for rapid gas exchange, while the root, due to its lack of stomata, presents a greater barrier to diffusion. Additionally, rubisco within the leaf is active at scavenging $CO_2$, while roots usually lack this enzyme. It seems probable that $CO_2$ levels

within the leaf would be influenced more by atmospheric $CO_2$ levels than the root. The root may already posses $CO_2$ concentrations near optimal for ACC oxidase activity, as a result of $CO_2$ release by respiration, and the slow diffusion of this $CO_2$ out of the organ.

A detailed investigation into the properties of ACC oxidase extracted from roots and leaves of both sunflower and corn shows that the properties of this enzyme are dependent on the organ it is extracted from. With the exception of the response of the enzyme to Fe, and the temperature optima, every other parameter examined showed significant differences between the root and the leaf.

Presumably the environmental conditions and physiological characteristics of the root and leaf have resulted in the unique adaptation of the root and leaf ACC oxidase activity. The root and leaf are exposed to very different gas compositions due to the different environments they inhabit. The root can be exposed to very high levels of $CO_2$ as a result of the respiration of plant material, bacteria and fungi, and the relatively low rate of gas diffusion in soil, especially wet soil. Soil $CO_2$ levels can easily reach 8% or greater [25], and the *in vivo* data presented here suggests that $CO_2$ levels within the root in the absence of atmospheric $CO_2$ may be high enough to saturate the enzyme. Conversely the leaf generally would not be exposed to $CO_2$ concentrations greater than .035%, and in fact the internal $CO_2$ concentration can be much lower due to $CO_2$ binding with rubisco. It is not surprising therefore to observe a divergence in the root and leaf ACC oxidase responses to $CO_2$. We observed here that the $K_{CO_2}$ for enzyme activity from the root was over 2–4 times higher than the leaf value in both corn and sunflower, which indicates that ACC oxidase activity from the leaf is more sensitive to the lower $CO_2$ levels present in its environment. Contrary to the case for $CO_2$, the leaf would generally be exposed to higher $O_2$ levels than the root, due to depletion of $O_2$ in the rhizosphere by respiration of components such as plant material, bacteria and fungi. Normally, $O_2$ levels in the atmosphere approximate 21%, while lower levels in the soil can be expected under certain conditions [26]. The $K_m$ for $O_2$ was found to be four times higher in corn leaves than roots, and over two times higher in sunflower leaves than roots. The greater affinity for $O_2$ shown by the ACC oxidase activity from the root might enable it to function more effectively at the lower $O_2$ levels present in the soil, especially during conditions contributing to anaerobiosis, such as flooding. Low $O_2$ conditions are known to stimulate ethylene production in both corn and sunflower roots leading to the formation of aerenchyma [27, 28], which may be important for plant survival in a hypoxic environment [29].

The ascorbate levels measured in the different tissues also corroborate the kinetic results obtained. In both sunflower and corn, ascorbate concentrations were lower in the roots than leaves, with a very large difference observed in sunflower. Corn leaves had a $K_m$ for ascorbate about 1.7 times the $K_m$ from corn roots, which corresponds well with the measured ascorbate levels from corn that showed leaves possessed about twice as much ascorbate as roots. The dramatic differences in sunflower ascorbate levels (leaves had about 30 times as much ascorbate as roots) were reflected in the greater difference in the affinity of leaf and root enzyme activities for ascorbate; the leaf $K_m$ for ascorbate was 4.5 times greater than the $K_m$ in roots. While the ascorbate levels measured might not accurately describe the concentration at the location of the enzyme, due to possible compartmentation, they probably reflect the relative levels of this substrate. Presumably the ACC oxidase activity is tailored to the ascorbate status of each organ.

The response of the ACC oxidase activity to ACC concentration was dissimilar between the two species (Table 1). When assayed at pH 6.5 both species appeared to behave the same, with the $K_{ACC}$ from the leaf ACC oxidase activity being about twice as high as the root $K_{ACC}$. When assayed at pH 6.8 however, the corn leaf $K_{ACC}$ dropped slightly below that of the corn root. While increasing the pH from 6.5 to 6.8 did reduce both sunflower organs' $K_{ACC}$, the leaf activity always had a $K_m$ about twice that of the root. ACC levels measured in leaves and roots showed that ACC was more concentrated in the leaves than in the roots of both species, but the differences were subtle. These results are complementary to corn as there is little apparent difference in the $K_m$s for ACC when assayed at optimal pH. The shift in $K_m$ observed in the corn activity with pH may reflect a divergence in the amino acids involved in binding ACC. It is possible that at the lower pH, a residue involved in binding ACC gains an unfavorable proton decreasing the affinity of the binding site for the substrate. Histidine would be an obvious candidate, as it has a p$K_r$ near 6.5 and is the only amino acid with a p$K_r$ near the conditions eliciting the effect. Using very different techniques Christoffersen *et al.* [30] have also implicated several histidine residues intimately associated with the active site of ACC oxidase. Possibly, differences in the number or location of histidine residues near the active site of the corn ACC oxidase could make the root enzyme less sensitive to changing pH than the leaf. This could also explain the reduction in $K_m$ for ACC observed in enzyme activity from both sunflower organs at high pH, assuming a similar distribution of histidines between root and leaf activities.

In conclusion, the data support the hypothesis that the root and leaf of both corn and sunflower possess organ specific ACC oxidase activity. Recent work illustrating tissue and stimulus specific ACC oxidase gene expression ([19], Liu and Reid, unpublished) supports the thesis of tissue specific ACC oxidase isoenzymes. Because the work presented was performed on crude extracts we cannot rule out the possibility of tissue specific interferences with the ACC oxidase assay. There is the possibility that a very tightly binding tissue specific ligand could alter the $K_m$ for the

enzyme activity, or that another tissue specific enzyme with the same substrate as ACC oxidase could complete with ACC oxidase and cause erroneous $K_m$ measurements. While these possibilities exist, the data suggest that the observed differences are not due to tissue specific interference. The fact that the behaviour of the enzyme activity was very similar between two widely divergent species supports the case for organ specific ACC oxidase activity. The very stringent test of linearity by the rigorous Eadie–Hofstee plots suggests that any competing enzymes would need very high affinities for a common substrate. Additionally we observe that the $K_m$ for some of the substrates were higher in the root, while others were higher in the leaf, and that the $K_{Fe}$ was the same in both organs of both species. These results argue against the presence of a tightly binding ligand, as this kind of interference would likely alter all the $K_m$s in the same direction. When we consider that the concentrations of substrates measured and the range of environmental gas levels presumed to exist for the two organs all correlate with the $K_m$s of the organ specific ACC oxidase activities, then taken together these observations are all consistent with the possibility of organ specific isoenzymes. The possibility also exists that the results obtained could be due to a tissue specific modifying protein interaction with the ACC oxidase.

## EXPERIMENTAL

*Plant material.* Sunflower (*Helianthus annuus* L. var. Dahlgren 131) seeds were germinated aeroponically with a 16 hr photoperiod of 220 $\mu$mol $m^{-2}$ $s^{-1}$ PAR under a 24/18° day/night temp. regimen. Seedling roots were maintained in the dark and misted with modified 25% Hoagland soln (double amount of iron chelate). The aeroponic germination system produced plants with completely undamaged roots. At 7 days after sowing, plants with leaves 2–3 cm long were selected. Corn (*Zea mays* L. cv. Texas 5855) was germinated on germination paper, then transferred to a hydroponic system with 10% Hoagland soln. The plants were grown with a 16 hr photoperiod of 365 $\mu$mol $m^{-2}$ $s^{-1}$ PAR under a 31/26° day/night temp. regimen in a system which prevented illumination of the roots. At 7 days after sowing plants with leaves 7–10 cm long were selected. Primary roots of corn were used throughout the experiments. Harvests were routinely conducted near the mid-point of the light period.

*Tissue ethylene evolution.* For analysis of $C_2H_4$ evolution from roots and leaves incubated with elevated $CO_2$, plants were grown as above, and entire roots and leaves were cut and incubated separately in 10 ml test tubes sealed with serum caps, containing 0–20% $CO_2$ and 21% $O_2$ (for 0% $CO_2$ 100 mg of KOH was included in the tube), under lighting as given above. After 20 min 1 ml of gas was withdrawn and analysed for $C_2H_4$ (wound $C_2H_4$ is not detectable in this system for at least 30 min). To assay the *in vivo* response of ACC oxidase to $CO_2$, roots of seedlings were immersed in 5 mM ACC in 25% Hoagland soln for 1 hr, under lighting as given above. Five roots or leaves were excised and placed together in 38 ml tubes sealed with serum caps with 0 or 5% $CO_2$ and 21% $O_2$. These tubes were incubated for 2 hr under light (as described for sunflower or corn) with 1 ml ethylene samples withdrawn and analysed at various times using a gas chromatograph equipped with a flame ionization detector. Tissue weights were determined at the end of the sampling period (for sunflower leaves *ca* 0.1 to 0.2 g fr. wt, roots 0.08 to 0.18 g fr. wt, corn leaves *ca* 0.15 to 0.3 g fr. wt, roots 0.1 to 0.25 g fr. wt). Since the early (less than 3 hr) $C_2H_4$ response to wounding appears to be a result of ACC synthase activity, no wounding effect could be observed in these experiments as the ACC treatment was apparently sufficient to saturate the ACC oxidase activity. These experiments were repeated at least 3 times.

In vitro *ACC oxidase extraction and assays.* ACC oxidase activity was extracted and assayed by a modification of the method of ref. [9]. One g of frozen tissue was ground under $N_2$ at 4° using a total of 4 ml of extraction buffer (100 mM BTP {1,3-bis[tris (hydroxymethyl)-methylamino]propane}–HCl, pH 7.0, with 10% glycerol and 2.5 mM DTT), and 5% (w/v) polyvinylpolypyrrolidone. The extract was centrifuged at 10000 *g* for 10 min, and the supernatant was reserved. Prior to conducting the assay, the extract was centrifuged again for 5 min at 10000 *g*.

Twenty $\mu$l of enzyme extract was injected into 6.6 ml tubes, sealed with rubber septa, containing 500 $\mu$l of assay buffer [100 mM BTP–HCl, pH 7.0 (giving a final pH of 6.8 due to acidification by $CO_2$), with 10% glycerol, 10 $\mu$M $FeSO_4$ 20 mM Na ascorbate and 5 mM ACC, pre-incubated with 20% $CO_2$, 21% $O_2$ for 20 min]. These tubes were incubated at 30° (sunflower) or 35° (corn) for 20 min on a shaking water bath. A 500 $\mu$l headspace sample was collected and $C_2H_4$ was analysed using a Photovac 10S10 or 10Splus GC equipped with a photoionization detector. Each parameter was varied separately, keeping all others constant. All assays were linear for the duration of the assay, and were repeated a minimum of 3 times. Because high concns of $CO_2$ acidify the buffer, assays examining the dependence of activity on $CO_2$ used buffers of varying pH giving a final pH of 6.8 at all $CO_2$ levels tested. $O_2$ levels were measured using a GC equipped with a thermal conductivity detector, for assays of enzyme activity with varying $O_2$ concentrations.

*Extraction and determination of ACC levels.* ACC levels in sunflower and corn roots and leaves were determined by isotope dilution and GC-SIM-MS. Fifty mg of tissue (taken from central part of roots and sunflower leaves, and from a cross section of expanding leaves above apex in corn) was weighed, frozen in liquid $N_2$, and then freeze dried. The extraction of ACC used ion exchange and HPLC as described earlier [31]. $^2H_4$-ACC was added as an

internal standard. ACC levels were determined by GC-SIM-MS using ions 244.7 and 248.7 for quantification. Three replicates of each tissue were measured.

*Determination of ascorbate and oxidized ascorbate.* Ascorbate and oxidized ascorbate were measured using a modification of the method of ref. [32]. Fresh 50 mg tissue samples (as above) were weighed into 1.5 ml microcentrifuge tubes and frozen and stored in liquid $N_2$. The tissue was homogenized in the tubes with 200 $\mu$l of 10% (w/v) meta-phosphoric acid (mpa) with 1% (w/v) $SnCl_2$. The extract was centrifuged at 10 000 *g* for 10 min and 100 $\mu$l of the supernatant was transferred to another tube. 150 $\mu$l of EtOAc was added, the tubes shaken and then centrifuged at 5000 *g* for 5 min. The EtOAc was removed, and 2–25 $\mu$l aliquots of the mpa extract were added to new tubes. To one of the tubes 50 $\mu$l of 1% (w/v) 2,6-dichlorophenol-indophenol was added, and left for 10 min on ice. This tube represented total ascorbate and oxidized ascorbate, the non-oxidized tube represented oxidized ascorbate only. For derivatization, 50 $\mu$l of 5% (w/v) mpa with 1% (w/v) $SnCl_2$ was added to all tubes, followed by 120 $\mu$l of 2% (w/v) 2,4-dinitrophenylhydrazine in 9N $H_2SO_4$. The tubes were kept at 35° for 2 hr to derivatize. After derivatization, 500 $\mu$l each of $H_2O$ and EtOAc was added. The tubes were centrifuged at 5000 *g* for 5 min and 300 $\mu$l of the EtOAc phase was removed and dried under $N_2$. The residue was redissolved in 600 $\mu$l of MeCN and a 20 $\mu$l aliquot was chromatographed isocratically in 55% (v/v) MeCN at 1 ml $min^{-1}$ on an HPLC with a $3.9 \times 150$ mm C–18 column. A photodiode array detector was used to scan the *A* from 192 to 650 nm, and the *A* at 505 nm was integrated for quantitation. Standard series of both ascorbate and dehydroascorbate were chromatographed following the experimental protocol exactly, and these showed a linear response in the region of experimental measurements. The percent recovery of ascorbate and dehydroascorbate for each tissue was determined by adding pure ascorbate or dehydroascorbate to homogenous samples, and comparing integrated areas to samples without added ascorbate to dehydro ascorbate and to standards. Analyses of ascorbate levels were performed twice, with six replicates of each sample.

*Statistics.* An ANOVA followed by a Fisher's test was calculated on the pH optimum curve using data from three experiments each. Data were ranked, with a total of 12 observations per point, and significance was determined at $\alpha = 0.05$. Differences in $K_m$s were accepted as significant when the 95% confidence intervals of the Eadie–Hofstee regressions did not overlap.

*Acknowledgements*—This work was supported by ATP grant no. 999902-087 to PWM and by a Natural Sciences and Engineering Research Council of Canada grant to DMR.

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