Inhibition and Stimulation of Root Respiration in *Pisum* and *Plantago* by Hydroxamate[1]

ITS CONSEQUENCES FOR THE ASSESSMENT OF ALTERNATIVE PATH ACTIVITY

RIES DE VISSER[*2] AND TJEERD BLACQUIÈRE
*Department of Plant Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren (Gn),
The Netherlands*

ABSTRACT

The contribution of the alternative pathway in root respiration of *Pisum sativum* L. cv Rondo, *Plantago lanceolata* L., and *Plantago major* L. ssp *major* was determined by titration with salicylhydroxamate (SHAM) in the absence and presence of cyanide. SHAM completely inhibited the cyanide-resistant component of root respiration at 5 to 10 millimolar with an apparent K_i of 600 micromolar. In contrast, SHAM enhanced pea root respiration by 30% at most, at concentrations below 15 millimolar. An unknown oxidase appeared to be responsible for this stimulation. Its maximum activity in the presence of low SHAM concentrations (1–5 millimolar) was 40% of control respiration rate in pea roots, since 25 millimolar SHAM resulted in 10% inhibition. In plantain roots, the maximum activity was found to be 15%. This hydroxamate-activated oxidase was distinct from the cytochrome path by its resistance to antimycin. The results of titrations with cyanide and antimycin indicated that high SHAM concentrations (up to 25 millimolar) block the hydroxamate-activated oxidase, but do not affect the cytochrome path and, therefore, are a reliable tool for estimating the activity of the alternative path *in vivo*. A considerable fraction of root respiration was mediated by the alternative path in plantain (45%) and pea (15%), in the latter because of the saturation of the cytochrome path.

The presence of the cyanide-resistant alternative pathway of electron transfer in mitochondria and intact tissues of many higher plant species is now well established (5, 11, 15, 23). However, the activity of this pathway *in vivo* is still a matter in dispute (5, 15, 20 *versus* 11, 13). Whatever the physiological significance, alternative path activity points to a lower ATP yield of oxidative phosphorylation than the maximum of 3 ATP/O. Therefore, determination of the *in vivo* activity is required for calculating the energy costs of metabolic processes in plants (7, 14).

To gain information on the activity of the alternative path in isolated mitochondria, hydroxamates are commonly used as inhibitors because of their specificity at low concentrations (1 mM range; 5, 13, 15, 22). Interpretation of inhibition by hydrox-

amates *in vivo*, however, is complicated by (a) the presence of other hydroxamate-sensitive oxidases (4, 20), and (b) the possibility that hydroxamate affects the activity of the Cyt path, *i.e.* by inhibition (13, 22, 27), or by diversion of electrons from the alternative path to an unsaturated Cyt path (5, 25). The latter possibility is supported by recent findings (6) indicating that the alternative path is engaged in the presence of an unsaturated Cyt path in roots of spinach, wheat, and maize. Inhibition of the alternative path which is attended with a lower rate of ATP production (17), will lead to an increased flux of electrons through the Cyt chain in such roots (5). The activity of the alternative path is thus underestimated by 33 to 100%, depending on the engagement of bypasses of site one (10, 16, 18) and/or site two (21, 26).

In this paper we examine the effects of a hydroxamate, SHAM[3], on root respiration of pea and plantain. We conclude that the Cyt path is not affected by SHAM. However, an unknown cyanide-sensitive oxidase appears to be present, which is activated by low hydroxamate concentrations and by an uncoupler, and is resistant to antimycin.

MATERIALS AND METHODS

Plant Material. Plants of *Pisum sativum* L. cv Rondo, *Plantago lanceolata* L., and *Plantago major* L. ssp *major* were grown in controlled environment cabinets in culture solutions as described previously (7, 12). Nutrients were supplied as described by Smakman and Hofstra (24). The temperature was 20°C. The light period was 12 h for *Plantago* and 16 h for *Pisum*. The age of the plants was 2 weeks for *Pisum* and 5 weeks for *Plantago*.

Respiration Measurements. Oxygen uptake by intact roots was measured polarographically with an YSI (Yellow Springs Instruments) model 53 oxygen monitor, as described by Lambers *et al.* (12). The composition of the nutrient solution was the same as during growth, except for the absence of iron, which chelates with SHAM. The temperature was 20°C. The pH was 6.0. Four (*Pisum*) or six (*Plantago*) intact root systems were used per measurement, unless indicated otherwise. Each titration curve was determined by successive addition of inhibitor to one set of roots. The maximum time required to complete one titration experiment was 1.5 h. Respiration rate of intact roots declined in the absence of inhibitor with 15%/h, due to a decreasing substrate supply to the electron transfer paths. The capacities of the electron transport paths did not change throughout the measurement. The nutrient solution was renewed when the

[2] Present address: Department of Plant Science, The University of Alberta, Edmonton, Alberta T6G 2P5 Canada.

[3] Abbreviations: SHAM, salicylhydroxamic acid; CCCP, carbonylcyanide *m*-chlorophenyl hydrazone; HOX, hydroxamate-activated oxidase.

oxygen concentration declined below 70% of air saturation. The effects of SHAM, cyanide, CCCP, and antimycin on root respiration rate stabilized within 5 min after addition of the inhibitor. Antimycin A and CCCP were dissolved in ethanol to give a stock solution of 50 and 2 mM, respectively. Ethanol did not affect respiration at the concentrations used in these experiments.

Analysis of Titration Data. Root respiration in the presence of various concentrations of SHAM was measured both in the absence and presence of KCN. For each SHAM concentration, rates of respiration in the presence of SHAM alone (v_T) were plotted against those obtained in the presence of both SHAM and KCN ($g(i)_{alt} + V_{res}$), essentially as described by Bahr and Bonner (3) for isolated mitochondria and Theologis and Laties (25) for potato slices. This ρ_{alt}-plot is described by the following equation:

$$v_T = \rho \cdot g(i)_{alt} + v_{cyt} + V_{res} \qquad (1)$$

where v_T is total respiratory activity which equals V_T, the total respiratory capacity, in case $\rho = 1$ and the Cyt path is saturated, v_{cyt} is the actual activity of the Cyt path and V_{res} is residual respiration, resistant to both cyanide and hydroxamate. The term $\rho \cdot g(i)$ represents the actual contribution of the alternative path in the absence of inhibitor, where $g(i)$ is the capacity of the alternative path as a function of the hydroxamate concentration, and ρ is a number between 0 and 1, being the fraction of the alternative path capacity which is actually engaged in root respiration.

To test if hydroxamate affects the activity of the Cyt path, root respiration was titrated with cyanide in the absence and presence of hydroxamate. At a range of given cyanide concentrations, the contribution of the Cyt path to root respiration in the absence of hydroxamate (v_T) was plotted against that in the presence of hydroxamate ($g(i)_{cyt} + V_{res}$). The slope of the resulting straight line gives ρ_{cyt}, i.e. the fraction of the Cyt path capacity which contributes to root respiration in the absence of inhibitors. This ρ_{cyt}-plot is described by:

$$v_T = \rho \cdot g(i)_{cyt} + v_{alt} + V_{res} \qquad (2)$$

The estimated value of ρ_{cyt} will be 1 if hydroxamate does not affect the activity of the Cyt path, <1 if hydroxamate stimulates, and >1 if hydroxamate inhibits the electron flow through the Cyt path.

Biochemicals. CCCP, SHAM, and antimycin A were obtained from Sigma Chemical Company.

RESULTS AND DISCUSSION

Effects of Hydroxamate on Root Respiration. Pea root respiration was stimulated 30% at most, by SHAM concentrations up to 15mM, even at concentrations which completely block the CN-resistant alternative path (5–10 mM; Fig. 1A). Consequently, the ρ_{alt}-plots were not linear (Fig. 1B). This was an unknown phenomenon in higher plant respiration. According to the generally accepted three-component model of intact tissue respiration (cf equation 1), the CN-sensitive, hydroxamate-activated oxidase must be identical with the Cyt path. This conclusion is unattractive for at least two reasons. First, it is unlikely that a stimulation of the Cyt path can exceed the inhibition of the alternative path by SHAM (see "Introduction"). Second, a direct activation of the Cyt path by hydroxamates has not been reported in respiration studies (5, 15, 22, 23). These findings and considerations lead us to investigate (a) the occurrence of a hydroxamate-activated oxidase in roots of other plant species, and (b) the nature and activity of the hydroxamate-activated oxidase.

Root respiration of two plantain species was titrated with SHAM. The presence of a hydroxamate-activated oxidase was demonstrated in roots of both *P. major* (Fig. 2) and *P. lanceolata* (Fig. 3), as shown most clearly by the ρ_{alt}-plots (Figs. 2B and 3B), and by the K_i values for SHAM in the absence and presence of cyanide (Table I). At concentrations below 5 mM, SHAM inhibited plantain root respiration less in the absence than in the presence of cyanide (P < 0.05).

Inactivation of cyanide by SHAM in wheat roots has been reported to cause non-linear ρ_{alt}-plots (13), but was essentially absent in roots of the present species. This is evident from the fact that residual respiration could not be blocked by the use of higher cyanide concentrations (1 mM) as shown for *P. lanceolata* (Fig. 3), which is unlike data of Lambers *et al.* (13) on wheat roots. Moreover, V_{res} in pea roots did not increase with the concentration of SHAM (Figs. 1A and 4A). Nonspecific effects of SHAM as observed in experiments with *Tetrahymena pyriformis* (28) were not apparent from our data on pea and plantain. We conclude that activation of a cyanide-sensitive oxidase by
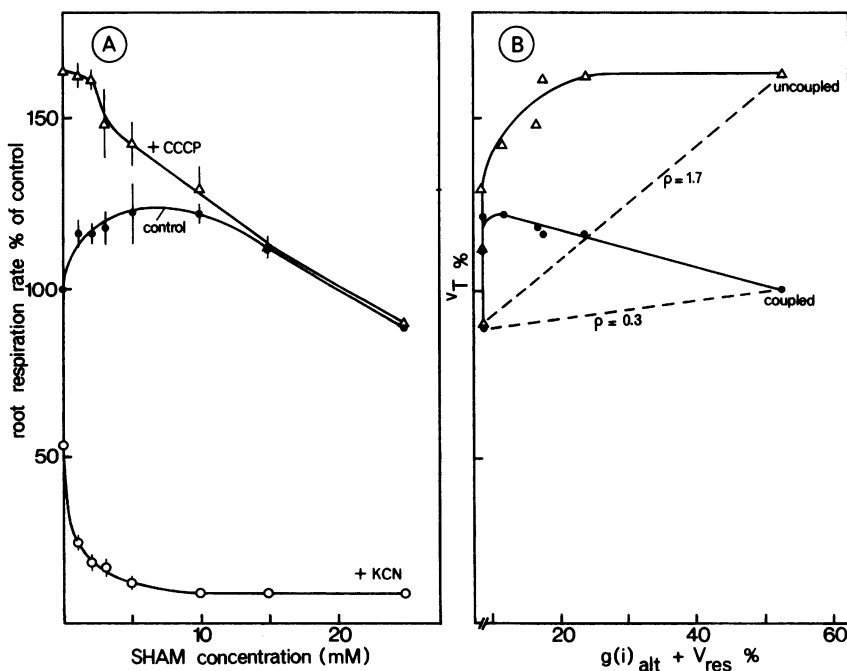

FIG. 1. Effect of SHAM on root respiration of *P. sativum* in the absence (●) and presence of 0.4 mM KCN (O) or uncoupler (2 μM CCCP, △). 100% equals 6.0 mg $O_2 \cdot h^{-1} \cdot g^{-1}$ dry roots. Each symbol is the mean of three to six independent determinations. Bars indicate 2 × SE (A). B, v_T as a function of $g(i)_{alt} + V_{res}$ (ρ_{alt}-plot); data from A.
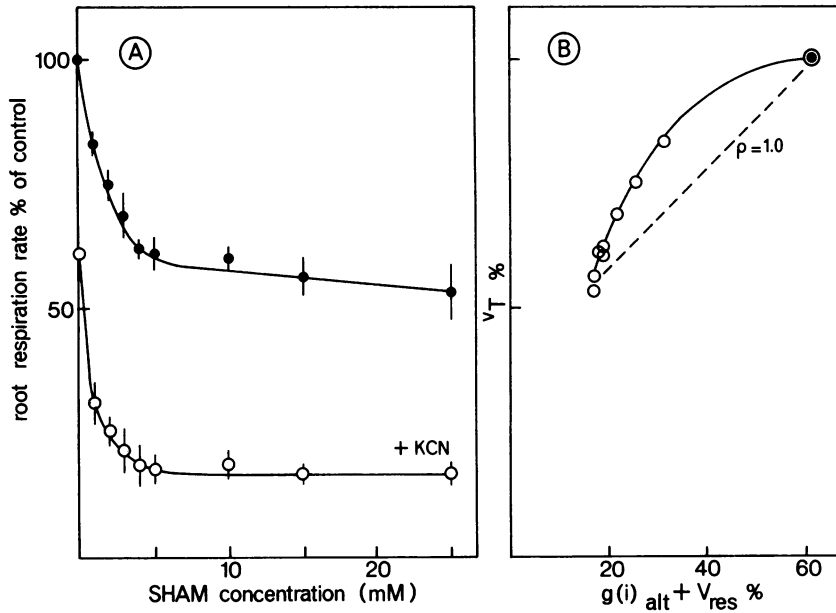
FIG. 2. Effect of SHAM on root respiration of *P. major* ssp *major* in the absence (●) and presence (○) of 0.4 mM KCN. 100% equals 7.3 mg $O_2 \cdot h^{-1} \cdot g^{-1}$ dry roots. Each symbol represents the mean of three independent determinations. Bars indicate 2 × SE (A). B, v_T as a function of $g(i)_{alt} + V_{res}$ (ρ_{alt}-plot); data from A.
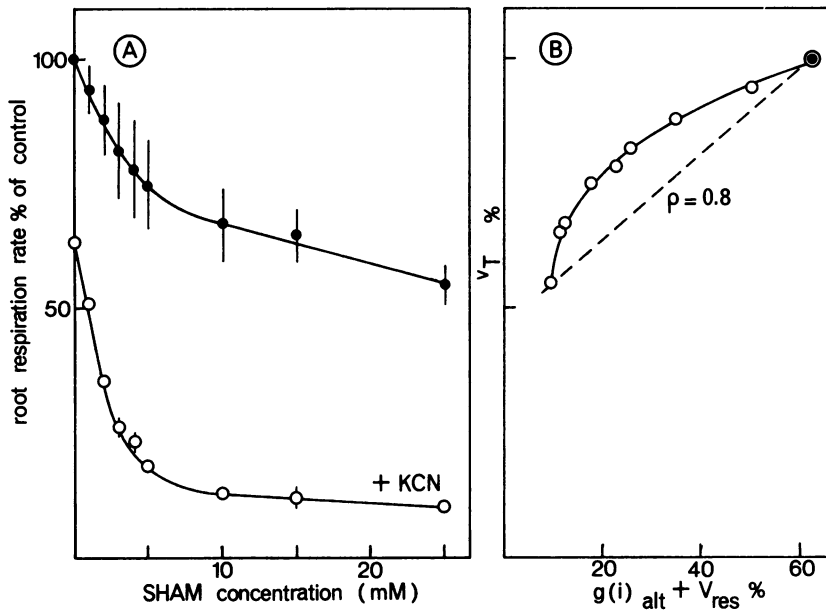

FIG. 3. Effect of SHAM on root respiration of *P. lanceolata* in the absence (●) and presence (○) of 1 mM KCN. 100% equals 7.5 mg $O_2 \cdot h^{-1} \cdot g^{-1}$ dry roots (A). B, ρ_{alt}-plot. For further information, see the legend to Figure 2.

Table I. *K_i Values for Inhibition of Root Respiration by SHAM and Cyanide*

Values for *P. sativum*, *P. lanceolata*, and *P. major* ssp *major* were estimated from Dixon-plots (8) of cyanide and SHAM titration data. Concentrations of KCN and SHAM were 0.4 and 25 mM, respectively.

Species	K_i of SHAM		K_i of Cyanide	
	Control	+ KCN	Control	+ SHAM
	mM		*μM*	
P. sativum	15.0	0.8	15	15
P. lanceolata	4.5	1.1	10	ND[a]
P. major	1.4	0.4	25	ND

[a] Not determined.

SHAM is the only factor causing nonlinearity of the ρ_{alt}-plots. In plantain roots, the extent of stimulation of the cyanide-sensitive oxidase by SHAM was less than the inhibition of the alternative path (Figs. 2 and 3), and was less than the stimulation of pea root respiration (Fig. 1). Therefore, hydroxamate effects on res-

piration were studied in detail on the latter tissue.

Nature of the Hydroxamate-Activated Oxidase (HOX). The SHAM-stimulated oxidase is characterized by the following properties. First, the oxidase is cyanide-sensitive (Figs. 1A, 2A, and 3A), with a K_i which is well within the range of K_i values reported for cyanide-sensitive respiration in wheat leaves (2), wheat roots (13), and tomato roots (9). Second, the oxidase is inhibited by high concentrations of SHAM, the K_i being one order of magnitude higher than the K_i of the alternative oxidase in pea roots (Table I). Third, the oxidase is stimulated by uncoupler (Fig. 1A), since the capacities of the Cyt and alternative paths and the residual respiration (80, 43, and 10% of the control) fail to account for the rate of uncoupled root respiration (163% of the control). The consequent suggestion that SHAM affects the activity of the Cyt path was scrutinized by titrating pea root respiration with cyanide in the absence and presence of 5 or 25 mM SHAM (Fig. 4). The results were analyzed using equation 2.

SHAM was equally effective in inhibiting cyanide-resistant respiration at 5 mM as well as at 25 mM. Root respiration showed the same cyanide-sensitivity, whether SHAM was absent or
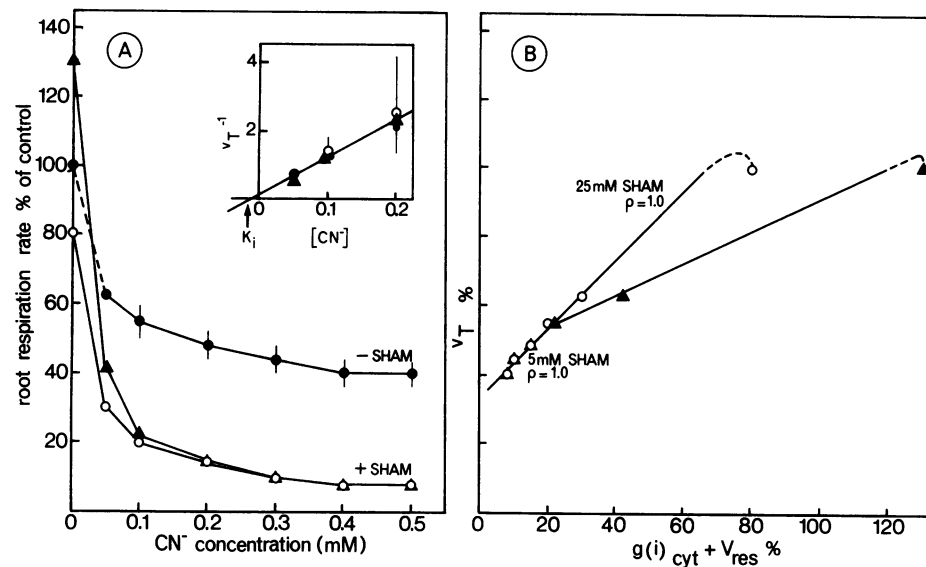
FIG. 4. A, Effect of cyanide (KCN) on the rate of root respiration of *P. sativum*, in the absence (●) and presence of 5 (▲) or 25 (○) mM SHAM. Each symbol represents one determination (+ SHAM) or the mean of three independent determinations (− SHAM). Bars indicate 2 × SE. 100% equals 6.0 mg $O_2 \cdot h^{-1} \cdot g^{-1}$ dry roots. Inset, Dixon-plot (8). B, v_T as a function of $g(i)_{cyt}$ + V_{res} (ρ_{cyt}-plot).
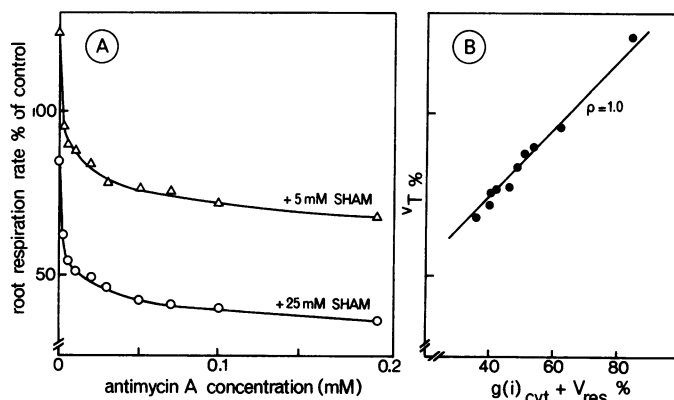

FIG. 5. Effect of antimycin A on root respiration of *P. sativum* in the presence of 25 mM SHAM (○) or 5 mM SHAM (△). Each symbol represents one determination with a set of two root systems (A). B, ρ_{cyt}-plot: antimycin-resistant respiration in the presence of 5 mM SHAM (v_T) as a function of that in the presence of 25 mM SHAM ($g(i)_{cyt}$ + V_{res}). Respiration rates in per cent of control rate (7.2 mg $O_2 \cdot h^{-1} \cdot g^{-1}$ dry roots).

present at 25 mM (ρ_{cyt} = 1.0; (Fig. 4B). According to the criterion formulated in "Materials and Methods," 25 mM SHAM had no detectable effect on the activity of the Cyt path. An important implication of this result is that 25 mM SHAM is enough for inhibiting HOX and, therefore, can be used for determination of alternative path activity *in vivo*. This could not be deduced from the SHAM titrations shown in Figure 1A. In addition, we have evidence that the Cyt path in pea roots was saturated, since uncoupler did not stimulate respiration in the presence of 25 mM SHAM (Fig. 1A). The alternative path was engaged (ρ_{alt} = 0.3) because the Cyt path was flooded with electrons. This situation is akin to that in bean roots (6). Both HOX and the alternative path became engaged upon uncoupling root respiration (Fig. 1A), presumably via a Pasteur effect (5).

However, 5 mM SHAM seemed to stimulate the Cyt path, as indicated by the ρ_{cyt} value lower than 1.0 in the cyanide concentration range up to 0.1 mM (Fig. 4B). This idea was tested by titrations with antimycin A. Figure 5 shows that antimycin-sensitive respiration equaled 50% of uninhibited respiration, whether the hydroxamate-activated oxidase was engaged (5 mM SHAM) or not (25 mM SHAM). The linear relationship between v_T and $g(i)_{cyt}$ + V_{res} (Fig. 5B), with a slope ρ_{cyt} equal to 1.0,

demonstrates that the engagement of Cyt path was the same in the presence of 5 and 25 mM SHAM. An uncoupler-like action of SHAM at low concentratios *in vivo* cannot be ruled out as yet (however, see below).

Summarizing, we conclude that the Cyt path in pea roots is not stimulated by low concentrations of hydroxamate, nor is it inhibited by high concentrations. Two possibilities remain. Hydroxamate may accelerate electron flow through the antimycin-resistant part of the Cyt chain which includes site 3 (5, 21). This is judged to be unlikely, since such a phenomenon has never been described for isolated mitochondria. On the other hand, hydroxamate may activate another, possibly nonmitochondrial oxidase. Three sources of evidence suggest that HOX is a peroxidase. First, some peroxidase reactions involve oxygen uptake (4). Second, peroxidase activity is present in pea roots (19). Third, the oxidation of ferrocyanide by horseradish peroxidase is stimulated by low and inhibited by high concentrations of benzhydroxamate (1).

Activity of the Hydroxamate-Activated Oxidase (HOX). The maximum activities of HOX, as calculated from the difference between the respiration rates in the presence of 5 and 25 mM SHAM, ranged from 35 to 45% (Figs. 1A, 4A, and 5A). Contribution of a significant part of this capacity of HOX to root respiration *in situ* would have been detected in the cyanide titration experiment (Fig. 4), yielding a ρ_{cyt} value higher than 1.0. Consequently, we conclude that HOX does not contribute to pea root respiration in the absence of inhibitors. Therefore, use of 25 mM SHAM appears to be a reliable tool for estimating alternative path activity in pea roots.

Activity of the Alternative Path. The extent of engagement of the alternative path, *i.e.* ρ_{alt}, was calculated as the quotient (inhibition by 25 mM SHAM):(resistance to cyanide minus V_{res}). In plantain roots, a high engagement of the alternative path was found (Figs. 2 and 3), in accordance with data on roots of cotton, tomato, and wheat (6, 13). A low alternative path engagement, as observed in pea roots (Fig. 1), has also been reported for other legumes (6, 13). Thus, evidence is presented that the alternative oxidase is not only present, but also active in intact roots, contributing a significant proportion to root respiration of pea (15%) and plantain (45%). In pea roots, the alternative path was operative since the Cyt path was saturated.

LITERATURE CITED

1. Aviram I 1981 The interaction of benzhydroxamic acid with horse radish peroxidase and its fluorescent analogs. Arch Biochem Biophys 212: 483–490
2. Azcón-Bieto J, H Lambers, DA Day 1983 The effect of photosynthesis and carbohydrate status on respiratory rates and the involvement of the alternative pathway in leaf respiration. Plant Physiol 72: 598–603
3. Bahr JT, WD Bonner Jr 1973 Cyanide-insensitive respiration. I. The steady states of skunk cabbage spadix and bean hypocotyl mitochondria. J. Biol Chem 248: 3441–3445
4. Butt VS 1980 Direct oxidases and related enzymes. In DD Davies, ed, The Biochemistry of Plants, Vol 2, Metabolism and Respiration. Academic Press, New York, pp 81–123
5. Day DA, GP Arron, GG Laties 1980 Nature and control of respiratory pathways in plants: The interaction of cyanide-resistant respiration with the cyanide-sensitive pathway. In DD Davies, ed, The Biochemistry of Plants, Vol 2, Metabolism and respiration. Academic Press, New York, pp 197–241
6. Day DA, H Lambers 1983 The regulation of glycolysis and electron transport in roots. Physiol Plant 58: 155–160
7. De Visser R, H Lambers 1983 Growth and the efficiency of root respiration of Pisum sativum as dependent on the source of nitrogen. Physiol Plant 58: 533–543
8. Dixon M 1953 The determination of enzyme inhibitor constants. Biochem J 55: 170–171
9. Janes HW, CK Chin 1981 The effect of age and growing conditions on cyanide resistance in cultured tomato roots. Plant Sci Lett 23: 307–313
10. Johnson-Flanagan AM, MS Spencer 1981 The effect of rotenone on respiration in pea cotyledon mitochondria. Plant Physiol 68: 1211–1217
11. Lambers H 1982 Cyanide-resistant respiration: A non-phosphorylating electron transport pathway acting as an energy overflow. Physiol Plant 55: 478–485
12. Lambers H, T Blacquière, CEE Stuiver 1981 Interactions between osmoregulation and the alternative respiratory pathway in Plantago coronopus as affected by salinity. Physiol Plant 51: 63–68
13. Lambers H, DA Day, J Azcón-Bieto 1983 Cyanide-resistant respiration in roots and leaves. Measurements with intact tissues and isolated mitochondria. Physiol Plant 58: 48–154
14. Lambers H, RK Szaniawski, R de Visser 1983 Respiration for growth, maintenance and ion uptake. An evaluation of concepts, methods, values and their significance. Physiol Plant 58: 556–563
15. Laties GG 1982 The cyanide-resistant, alternative path in higher plant respiration. Annu Rev Plant Physiol 33: 519–555
16. Marx R, K Brinkman 1978 Characteristics of rotenone-insensitive oxidation of matrix-NADH by broad bean mitochondria. Planta 142: 83–90
17. Mercier PJ, RJ Poole 1980 Electrogenic pump activity in red beet: Its relation to ATP levels and to cation influx. J Membr Biol 55: 165–174
18. Møller IM, JM Palmer 1982 Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner surface of the inner membrane of plant mitochondria. Physiol Plant 54: 267–274
19. Oostrom H, FE Treurniet, AM Mennes 1975 Cytochemical localization of peroxidase during the development of root nodules of Pisum sativum L. Z Pflanzenphysiol 74: 451–463
20. Rich PR, NK Wiegand, H Blum, AL Moore, WD Bonner 1978 Studies on the mechanism of inhibition of redox enzymes by substituted hydroxamic acids. Biochim Biophys Acta 525: 325–337
21. Rustin P, F Moreau, C Lance 1980 Malate oxidation in plant mitochondria via malic enzyme and the cyanide-insensitive electron transport pathway. Plant Physiol 66: 457–462
22. Schonbaum GR, WD Bonner, BT Storey, JT Bahr 1971 Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Plant Physiol 57: 124–128
23. Siedow JN 1982 The nature of the cyanide-resistant pathway in plant mitochondria. In LL Creasy, G Hrazdina, eds, Recent Advances in Phytochemistry, Vol 16, Cellular and Subcellular Localization in Plant Metabolism. Plenum Press, New York, pp 47–83
24. Smakman G, JJ Hofstra 1982 Energy metabolism of Plantago lanceolata as affected by change in root temperature. Physiol Plant 56: 33–37
25. Theologis A, GG Laties 1978 Relative contribution of cytochrome-mediated and cyanide resistant electron transport in fresh and aged potato slices. Plant Physiol 62: 232–237
26. Theologis A, GG Laties 1978 Antimycin-insensitive cytochrome-mediated respiration in fresh and aged potato slices. Plant Physiol 62: 238–242
27. Van der Plas LHW, MJ Wagner 1980 Influence of ethanol on alternative oxidase in mitochondria from callus-forming potato tuber discs. Physiol Plant 49: 121–126
28. Young PG 1983 The SHAM-sensitive alternative oxidase in Tetrahymena pyriformis: Activity as a function of growth state and chloramphenicol treatment. J Gen Microbiol 129: 1357–1363