Effects of KCN and Salicylhydroxamic Acid on Respiration of Soybean Leaves at Different Ages

ABSTRACT

Measurements of respiration were made on leaf discs from glasshouse-grown soybean (Glycine max [L.] Merr. cv 'Corsoy') plants in the presence and absence of cyanide (KCN) and salicylhydroxamic acid (SHAM). O₂ uptake by mature leaves measured at 25°C was stimulated by 1 millimolar KCN (63%) and also by 5 millimolar azide (79%). SHAM, an inhibitor of the alternative oxidase and a selection of other enzymes, also stimulated O₂ uptake by itself at concentration of 10 millimolar. However, in combination, KCN and SHAM were inhibitory. The rate of O₂ uptake declined consistently with leaf age. The stimulation of O₂ uptake by KCN and by SHAM occurred only after a certain stage of leaf development had been reached and was more pronounced in fully expanded leaves. In young leaves, O₂ uptake was inhibited by both KCN and SHAM individually. The uncoupler, p-trifluoromethoxy carbonyl-cyanide phenylhydrazone, stimulated leaf respiration at all ages studied, the stimulation being more pronounced in fully expanded leaves. The uncoupled rate was inhibited by KCN and SHAM individually. The capacity of the cytochrome pathway declined with leaf age, paralleling the decline in total respiration. However, the capacity of the alternative pathway peaked at about full leaf expansion, exceeding the cytochrome capacity and remaining relatively constant. These results are consistent with the presence in soybean leaves of an alternative pathway capacity that seems to increase with age, and they suggest that the stimulation of O₂ uptake by KCN and NaN₃ in mature leaves was mainly by the SHAM-sensitive alternative path. The stimulation of O₂ uptake by SHAM was not expected, and the reason for it is not clear.

The presence of a cyanide-resistant alternative respiratory pathway in mitochondria and intact plant tissue is routinely concluded from an insensitivity of O₂ uptake to cyanide and a specific inhibition by hydroxamates in the presence of cyanide (7, 15, 18). Its presence or activity has been demonstrated in a wide variety of plant tissues, and the proportion of a plant's respiratory capacity that is cyanide-resistant has been shown to vary among species and from tissue to tissue (7, 15, 18). Aroids (21) have received much attention because of the unusually high level of cyanide-resistant respiration in their spadices. Storage organs are of interest because this pathway is either induced or regenerated in aged slices (18, 27).

Studies of leaf respiration, using both isolated mitochondria and intact tissues, have provided a range of results. The pattern of leaf respiration has been shown to change with leaf development and there are indications that the degree of resistance to cyanide, and therefore, the possible contribution of the alternative pathway to total respiration, is either constant (1) or increases with maturation (7). O₂ uptake rates in bean (Phaseolus vulgaris) leaves decreased by about 70% by d 14 (1). A similar decrease in leaf respiration with maturation had been reported earlier (11, 14). This decline in leaf respiration has been attributed to the degeneration and consequent decrease in the biochemical efficiency of the mitochondria (11, 26). Earlier reports indicated that, whereas the respiration of young leaves is inhibited, that of mature leaves is either resistant or even stimulated by inhibitors of the Cyt oxidase (19, 20). It is noteworthy, however, that these earlier observations of respiratory stimulation were made before the discovery of specific inhibitors of the alternative pathway and therefore do not provide complete information regarding the possible involvement of the alternative pathway in these responses.

The present study is part of an effort to characterize alternative pathway respiration in soybean tissues. Published measurements of cyanide-resistant or alternative pathway respiration in soybean tissues are sparse and almost invariably have been restricted to imbibing seeds (24, 29), embryonic axes (23), and cultured cells (8, 22). Hubiec et al. (13) reported a 30% cyanide resistance in mitochondria isolated from young leaves, with malate and glutamate as substrates and with no residual respiration. To our knowledge, this is the only published report in the current literature on the involvement of the alternative pathway in soybean leaf respiration. In this report, we present results indicating stimulation of O₂ uptake by respiratory inhibitors in mature soybean leaves, and which suggest the existence of an alternative pathway capacity that increases with leaf maturation.

MATERIALS AND METHODS

Plant Material. Soybean (Glycine max [L.] Merr. cv 'Corsoy') plants were grown in a glasshouse in 25-cm plastic pots containing either a 2:1:1 mixture of soil, peat, and sand or a 1:1:1 mixture of soil, sand, and perlite. Day temperatures ranged from 30 to 35°C and, night, from 20 to 25°C. Supplemental radiation was supplied by high pressure sodium lamps providing a 14-h photoperiod. One week after emergence, seedlings were thinned to one plant per pot. Plants were watered daily with tap water and flooded once a week with a solution of a general purpose (20-20-20) commercial fertilizer (Ceres Fertilizer Products). Plant position on the benches was altered weekly to minimize any possible glasshouse positional effects.

Sampling. Leaves of either the third or the fifth node were


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were calibrated against course the solution presented a distilled FCCP dry-weight stimulated SHAM, and stimulation by separate three methoxyethanol. After ing an applications leaflets reached full out, the laboratory 444 SESAY 5 ml of distilled H2O. Of the leaflets were rinsed repeatedly with chilled distilled H2O. In most cases, only the terminal leaflets were used. Leaflets from three separate plants were pooled per replication, and four replications were used.

Leaf age was calculated in days, and ‘day zero’ was established as the day on which the leaflets of the selected leaf unfolded. The leaflets reached full expansion in 8 to 9 d.

Measurement of Respiratory Characteristics. Discs, each having an area of 0.3 cm², were punched from the sampled leaflets with a stainless steel punch. The discs were rinsed in chilled distilled H2O. Twenty leaf discs were transferred to a transparent glass cuvette. After 15 min temperature equilibration at 25°C in 5 ml distilled H2O, the rate of O₂ uptake (control rate) was measured using Clark-type O₂ electrodes and Yellow Spring Instruments Model 53 O₂ meters. Inhibitors were then added and the discs stirred for 25 to 30 min. Maximal inhibition or stimulation by either KCN or SHAM appeared gradually over the course of a 25 to 30 min period. The solution was aerated, then the rates in the presence of the inhibitors were determined.

FCCP rates stabilized in about 3 to 5 min. Similar results were obtained in preliminary studies when distilled H2O or buffered solution (1) was used as the measuring medium. The electrodes were calibrated against air-saturated distilled H2O. KCN, NaN₃, SHAM, and FCCP were used at final concentrations of 1 mm, 5 mm, 10 mm, and 1 µM, respectively. KCN was dissolved in distilled H2O, NaN₃, FCCP in 95% ethanol, and SHAM in 2-methoxyethanol. After measurements, samples were oven-dried at 80°C for at least 48 h to allow expression of the rate of O₂ uptake per g dry weight.

RESULTS

O₂ uptake rates of leaf discs of mature soybean leaves are presented in Table I. O₂ uptake, expressed either on a leaf-area or dry-weight basis, was strongly stimulated by KCN (63%) and NaN₃ (79%). Quite unexpectedly, SHAM, an inhibitor of the alternative path and a selection of known enzymes (4, 16), also stimulated O₂ uptake by itself (36%). However, KCN in combi-

<table>
<thead>
<tr>
<th>Sequential Additions</th>
<th>Rate of O₂ Uptake</th>
<th>Stimulation (+) or Inhibition (−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol m⁻² s⁻¹</td>
<td>µmol g⁻¹ dry wt min⁻¹</td>
</tr>
<tr>
<td>Experiment 1:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.57 ± 0.07</td>
<td>2.23 ± 0.29</td>
</tr>
<tr>
<td>+ KCN</td>
<td>0.93 ± 0.09</td>
<td>3.64 ± 0.34</td>
</tr>
<tr>
<td>+ KCN + SHAM</td>
<td>0.25 ± 0.04</td>
<td>0.99 ± 0.16</td>
</tr>
<tr>
<td>Experiment 2:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.59 ± 0.04</td>
<td>2.30 ± 0.23</td>
</tr>
<tr>
<td>+ SHAM</td>
<td>0.80 ± 0.05</td>
<td>3.12 ± 0.21</td>
</tr>
<tr>
<td>+ SHAM + KCN</td>
<td>0.25 ± 0.06</td>
<td>0.98 ± 0.20</td>
</tr>
<tr>
<td>Experiment 3:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.01 ± 0.18</td>
<td>3.01 ± 0.23</td>
</tr>
<tr>
<td>NaN₃</td>
<td>1.81 ± 0.14</td>
<td>5.40 ± 0.54</td>
</tr>
</tbody>
</table>

Abbreviations: SHAM, salicylhydroxamic acid; FCCP, p-trifluoromethoxy carbonylcyanide phenylhydrazone; V₅₃, alternative pathway capacity; V₆₃, cytochrome pathway capacity.

The presence of FCCP did not alter the general pattern of respiratory decline during leaf maturation. The reason for the more precipitous drop in the control rates in Figure 4 compared to Figures 1 to 3 is not apparent except to note that different
batches of plants were used in the studies reported in the two groups of figures. The results shown in Figure 4 are summarized in Table II. It should be pointed out that while the values reported for the capacity of the Cyt path (V_cyt) in Table II may be of some theoretical interest, they should be regarded only as approximations. The capacity of the Cyt path would normally be estimated as the rate of respiration in the presence of an uncoupler and SHAM minus the residual rate. This method was considered unsuitable in this case given the unusual effect of SHAM in the presence of FCCP (Fig. 5). The capacity of the Cyt path was therefore estimated as inhibition by KCN in the presence of the uncoupler FCCP. This method was based on the assumption, perhaps unjustified, that uncoupling with FCCP led to a respiration rate that fully saturated both pathways or at least approached saturation. In the absence of an absolute way of ascertaining the maximal rate of electron transport (17), and because the alternative path is engaged only when the Cyt path is saturated or restricted (2, 18, 27), O_2 uptake rates in the presence of FCCP were taken as representing the maximum respiratory capacity. Day and Lambers (6) observed that in bean, wheat (Triticum aestivum L.), and maize (Zea mays L.) the potential respiratory rates, measured with isolated mitochondria, were similar to the root rates measured in the presence of FCCP. It seems of interest that the decline in total respiration with leaf age was closely paralleled by a decrease in the estimated capacity of the Cyt path (V_cyt). In contrast, the capacity of the alternative path (V_alt) peaked at about full leaf expansion, exceeding V_cyt. Thus, the ratio V_cyt/V_alt declined with leaf maturation.

**DISCUSSION**

The results obtained from the present study indicate that O_2 uptake by mature soybean leaf tissue is stimulated by inhibitors
of both the Cyt and alternative-path terminal oxidases. The stimulation of respiration by inhibitors of Cyt oxidase has been observed previously for mature leaves of carrot, Daucus carota (20), mustard, Sinapis alba, and belladonna, Atropa belladonna (19). The interaction between KCN and SHAM in inhibiting O₂ uptake by soybean leaf tissue is consistent with the presence of an alternative path (5, 7, 16) and further suggests that KCN and NaN₃ were stimulating O₂ uptake mainly by the SHAM-sensitive alternative path. The stimulation of O₂ uptake by SHAM alone, as far as we know, is an unknown phenomenon for leaf tissue, although it has recently been observed in roots of other legume plants (9). Propyl gallate, a potent inhibitor of alternative oxidase, also stimulated O₂ uptake (data not shown).

According to existing information, cyanide stimulation of O₂ uptake should occur only in tissues with a large potential capacity for the alternative path. To estimate the relative potential capacities of the pathways during leaf development, the effect of KCN and SHAM in the presence of the uncoupler FCCP was determined. The effect of FCCP is to abolish the electrochemical proton gradient across the inner mitochondrial membrane and thereby allow maximum electron flow through the respiratory pathways (10). Inhibition of O₂ uptake by either KCN or SHAM in the presence of the uncoupler would therefore allow the capacities of the pathways to be estimated.

It seems quite likely that our results with FCCP and SHAM (Fig. 5) were influenced by some unspecific inhibitor action. A number of considerations complicate the interpretation of inhibitory effects of hydroxamates in vivo. Notable among these considerations are: (a) the presence of other hydroxamate-sensitive oxidases (4, 16), and (b) the possibility of other effects of hydroxamates on metabolism (9, 16). In this study, SHAM seemed to have some specificity for the alternative path as judged by its strong inhibition in the presence of KCN. However, the stimulation of O₂ uptake in mature leaves by SHAM alone would require either that SHAM causes the diversion of electron flux from the alternative path into an unsaturated Cyt path, an effect believed to be improbable (9, 27), or that SHAM has an additional effect either of uncoupling or of activation of extramitochondrial enzymes (9, 16). Along these lines, it is of interest to note that Lambers and his colleagues (15) have shown that high concentrations of SHAM (10–20 mM) can safely be used and even are required to give full inhibition of the alternative path in intact leaves and roots. De Visser and Blacquiere (9) observed SHAM simulation of respiration at low concentrations in roots of Pisum sativum and Plantago species, whereas they observed an inhibition at higher SHAM concentrations (25 mM). In titration experiments, we failed to detect inhibition of respiration by SHAM in the absence of KCN, even at SHAM concentrations as high as 30 mM (data not shown). However, there was a consistent decline in the stimulatory effect of SHAM at concentrations above 10 mM, and at 30 mM the stimulatory effect was completely lost. Maximal inhibition of respiration by SHAM in the presence of KCN was obtained at 10 mM.

The effect of KCN on O₂ uptake in the presence of FCCP gives information on the relative capacities of the two electron

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Table II. Estimation of the Capacities of Respiration Pathways in Soybean Leaves

<table>
<thead>
<tr>
<th>Leaf Age</th>
<th>Vₚ</th>
<th>Respiratory Capacities</th>
<th>Vₚ/Vₐt ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/g dry wt·min⁻¹</td>
<td>Vₚ</td>
<td>Vₐt</td>
</tr>
<tr>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.12</td>
<td>3.28</td>
<td>0.98</td>
</tr>
<tr>
<td>6</td>
<td>1.02</td>
<td>1.31</td>
<td>1.54</td>
</tr>
<tr>
<td>10</td>
<td>1.04</td>
<td>0.72</td>
<td>1.30</td>
</tr>
<tr>
<td>14</td>
<td>1.10</td>
<td>0.91</td>
<td>1.36</td>
</tr>
<tr>
<td>18</td>
<td>0.42</td>
<td>0.30</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Rates shown were calculated from the data of Figure 4. Residual respiration (O₂ uptake in the presence of FCCP + KCN + SHAM) was subtracted from all values. Vₚ is the rate of O₂ uptake in the absence of FCCP and inhibitors. Vₚₘ₆₄ stands for the capacity of the Cyt path, estimated as inhibition in the presence of FCCP and KCN. Vₚₘ₆₄ is the capacity of the alternative pathway, estimated as O₂ uptake in the presence of FCCP and KCN.

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Fig. 5. Effect of SHAM on respiration of soybean leaf discs in the presence of 1 μM FCCP as a function of leaf age. For further information see legend of Figure 1.
transport pathways. The increase in \( V_{\text{at}} \) occurred at the time of \( O_2 \) uptake stimulation by KCN and also when the stimulation by FCCP was most marked (Fig. 4). An important implication of these observations is that the rate of electron flux through the alternative path can exceed the maximum activity or capacity of the Cyt path in soybean leaf tissue. Thus, it might seem that the stimulation of \( O_2 \) uptake in mature soybean leaves in the presence of KCN and NaCN could be explained by the scheme put forward for mature Chlorella cells (12). This explanation is based on the assumption that the glycolytic rate is limited by the availability of phosphate acceptors (5) and, therefore, that the respiratory pathways are not saturated in the absence of inhibitors. This assumption is supported in this study by the stimulation of \( O_2 \) uptake by the uncoupler FCCP, although uncoupler stimulation could also conceivably result from the relief of respiration restraint in the electron transport path (3). It is clear, however, that the latter alternative would not lead to the observed results. The uncoupler was less effective in the young leaves, a response that is expected of young growing tissues. Day et al. (7) observed a similar response in Lolium. Presumably, with the high rate of \( O_2 \) uptake in young tissues, respiratory flux was already near maximal. This probably explains why the young leaves were sensitive to the inhibitors.

According to the proposed scheme for the stimulation of \( O_2 \) uptake in mature soybean leaves in the presence of KCN and NaCN, the inhibition of the Cyt path, which is coupled to phosphorylation, diverts electron flow through the alternative path, thereby circumventing respiratory control. With the increased capacity of the alternative path, the increased electron flow can be accommodated, so total \( O_2 \) uptake is increased. A major difficulty with this scheme is the need to demonstrate that the apportioning of electrons to the alternative path is responsible for the respiratory stimulation. According to present thinking, however, the fact that the stimulation of \( O_2 \) uptake by KCN and NaCN is abolished by SHAM seems to provide good, though equivocal, evidence. The situation is further clouded by the consideration that, if the rotenone-sensitive NADH dehydrogenase and the alternative path operate in series, then electron transport through complex I (endogenous NADH to ubiquinone) would remain under respiratory control in the presence of KCN, since electrons branch to the alternative path from ubiquinone. It would seem therefore that the complete circumvention of respiratory control would require either that complex I must also be bypassed, a possibility inherent in the rotenone-resistant respiration so prevalent in plant tissues but a possibility on which some doubt has been cast (28), or that complex I must be disabled in some way in the presence of KCN. Thus, it is conceivable that while the alternative pathway itself is nonphosphorylating, the oxidation of tricarboxylic acid cycle substrates via the path in the presence of KCN may not be totally nonphosphorylating, at least for a certain period of time. However, even with the indication of a large potential capacity of the alternative path, our results did not indicate engagement of the alternative path in the absence of KCN, as shown by the stimulation rather than inhibition of \( O_2 \) uptake by SHAM alone. It remains to determine the relevance of the large alternative path capacity in mature soybean leaves.

LITERATURE CITED


3. BISHOP PD, DE ATKINSON 1984 Adenine nucleotide control of the rate of oxygen uptake by rate heart mitochondria over a 15- to 20-fold range. Arch Biochem Biophys 230: 335–344


