Regulation of Respiration in the Leaves and Roots of Two *Lolium perenne* Populations with Contrasting Mature Leaf Respiration Rates and Crop Yields

Received for publication December 18, 1984 and in revised form March 26, 1985

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**ABSTRACT**

Measurements of \( O_2 \) uptake were made on leaves and roots of two populations of *Lolium perenne* L. cv S23 (GL66 and GL72), previously shown to have contrasting rates of CO\(_2\) evolution and yields of dry matter. \( O_2 \) uptake was faster in the mature leaves of GL66 than those of GL72, but no difference was observed in the respiratory rates of meristem leaf bases or mature roots. The growth rate of GL72 was faster than that of GL66. Cyanide resistance was substantial in mature leaves but the alternative path did not contribute to \( O_2 \) uptake in the dark. In both populations, adding malate and glycine stimulated \( O_2 \) uptake, but exogenous sucrose only stimulated when uncoupler was also present. The difference between the respiratory rates of the two populations was maintained under all investigated conditions. We conclude that the rate of mature leaf respiration in the dark in *L. perenne* is limited by adenylate control of glycolysis. The difference between the fast (GL66) and slow (GL72) respiring populations reflected a greater respiratory capacity and higher turnover of ATP in GL66. Alternative path capacity was also high in the roots of both and contributed substantially to \( O_2 \) uptake, as indicated by inhibition by salicylhydroxamic acid in the dark, and not with the rate of photosynthesis or photorespiration. This increase in biomass was positively correlated only with the rate of mature leaf respiration measured as CO\(_2\) loss in the dark, and not with the rate of photosynthesis or photorespiration, and was not associated with any obvious agronomically undesirable characteristics.

To date, nothing is known concerning the biochemical nature or regulation of respiration in these plants, although such knowledge is obviously important for future exploitation of respiratory parameters and the detection of possible deleterious side effects of selection for slower respiring lines. In the present paper, we report initial investigations of leaf and root respiration in two *L. perenne* populations previously shown to differ in their rates of dark CO\(_2\) efflux from mature leaves. Particular attention was paid to the relative involvement of the non-energy conserving, alternative pathway of electron transport, since this might be expected to affect the respiratory efficiency. Some anatomical investigations of the leaves were carried out, in an attempt to find clues to the nature of the greater turnover of ATP which was indicated by the results of biochemical studies on the respiration of the *Lolium* lines.

**MATERIALS AND METHODS**

**Seed Production and Growth of Plants.** Two populations derived from *Lolium perenne* cv S23 were examined. One, GL66, was a polycross progeny obtained by open pollination of nine genotypes selected for fast mature leaf respiration. The other, GL72, was a polycross progeny similarly obtained from 18 genotypes selected for slow respiration (15, 16). GL72 was derived from the same slow respiration families as another population, GL83, whose growth in the field is described by Wilson and Jones (17). Seeds were germinated in vermiculite and the seedlings transferred into nutrient solution (14) about 10 d after sowing. Growth occurred in a controlled environment cabinet (20°C, 16 h d, 250 \( \mu \)mol m\(^{-2}\) s\(^{-1}\), photosynthetically active radiation). The age of the plants used varied from 15 to 90 d.

**Measurements of \( O_2 \) Consumption.** Leaves. Small segments were cut with a razor blade from either the middle portion of fully expanded leaves (mature tissue: 2-mm segments were cut) or from the unexposed bases of young leaves (meristematic tissue: 5-mm segments were cut). For each separate measurement, at least four different leaves were used and cutting was done with the leaves submerged in 20 mm Hepes buffer (pH 6.5), containing 0.2 mm CaCl\(_2\). The slices were incubated in the buffer for 30 min...
at room temperature (20–25°C) in the dark, to avoid possible interference from postillumination transients (2). O₂ uptake was measured at 20°C in 5 ml of the above buffer using a Clarke type of O₂ electrode (YSI, Yellow Springs, OH). When effector solutions were used, they were added directly to the reaction vessel, and O₂ uptake was subsequently followed for 10 to 15 min. SHAM* was added from a 1 M solution in methoxy-ethanol; CCCP from a 2 mM solution in ethanol.

Roots. For each measurement, all the intact roots of three different plants were cut and thoroughly rinsed in nutrient solution (see above). O₂ uptake was measured in 100 ml of nutrient solution (minus Fe) at 20°C in a sealed cuvette with a Clarke O₂ electrode. When the effect of SHAM was investigated, the original solution was replaced by a similar one containing 15 mM SHAM. KCN and CCCP were added directly to the vessel. After measurements, the roots were blotted dry and weighed.

Estimation of Cyt Oxidase and Alternative Path Activity. Previous results from our laboratories have shown that the contribution of the respective pathways to overall O₂ consumption in leaves and roots can be estimated using KCN to inhibit Cyt oxidase and SHAM to inhibit the alternative path (3, 6, 7). Inhibition of KCN allows the capacity (maximum possible activity) of the alternative path to be estimated, while inhibition by SHAM alone indicates the actual activity of the alternative path (i.e., its contribution to O₂ uptake). Since the alternative path acts as an overflow of the Cyt path—it is only engaged when the Cyt path is saturated, or inhibited by lack of ADP (8, 10)—inhibition by SHAM in the presence of uncoupler allows the capacity of the Cyt path to be estimated. On the other hand, inhibition by SHAM in the absence of uncoupler but not in its presence, shows that adenylate control of the Cyt chain occurs (2). Lack of inhibition of O₂ uptake either in the absence or in the presence of uncoupler indicates that the alternative path does not operate.

Chemical Analyses. Sugars (glucose plus fructose) were assayed enzymatically in dried and ground material (1). Nitrogen analyses were done with the Kjeldahl method, using CuSO₄-K₂SO₄ (1:3) as a catalyst (5). The samples were partitioned into a 3% HCl soluble fraction and an insoluble fraction.

RESULTS

Leaf Respiration and Plant Growth. Table 1A shows average rates of O₂ uptake in the dark by slices cut from fully expanded leaves of the two populations. In agreement with previously reported rates of CO₂ evolution from whole attached leaves (12, 13), GL66 (the ‘fast’ respiring line) yielded rates approximately 40% faster than those of GL72 (the ‘slow’ respiring line). Neither KCN (an inhibitor of Cyt oxidase), nor SHAM (an inhibitor of the alternative path) had any significant effect on the rate of O₂ uptake by mature leaves from either line when added alone, but when these inhibitors were added together, respiration was severely inhibited (Table 1A). The slower respiration rate of GL72 was reflected in the growth of the plants; the increase in dry weight of both shoots and roots with plant age was greater in GL72 than in GL66 (Fig. 1A), and fully expanded leaves were both longer and thicker in GL72 (see below). The shoot to root ratio was virtually identical in the two populations (Fig. 1B). Initial relative growth rate was also significantly higher in GL72 (219 versus 186 mg·g⁻¹ [plant dry weight] for GL66). This agrees with the previous analyses of Robson (12, 13).

The pattern of respiration in the meristematic bases of leaves was quite different from that in the mature leaf (Table 1B). The rate of O₂ uptake by slices from meristematic regions was the same in both populations, and this O₂ uptake was severely inhibited by KCN alone. Addition of SHAM alone had no effect on O₂ uptake (Fig. 2D) but when added after KCN it caused a further inhibition (Table 1B). These differences between mature and meristematic leaf tissue can be seen more clearly in Figure 2 which depicts typical individual O₂ traces. Although only results obtained with GL72 are shown, similar trends were observed with GL66. O₂ uptake by slices taken from the middle of fully expanded leaves was slower than that by slices from unexposed (yellow) leaf bases and was unaffected by SHAM and only

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* Abbreviations: SHAM, salicylhydroxamic acid. CCCP, carbonylcyanide m-chlorophenyl hydrazone.

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Table 1. Mean Respiratory Rates of Leaves of Two Populations of L. perenne, Selected for Either Fast (GL66) or Slow (GL72) Mature Leaf Dark Respiration

<table>
<thead>
<tr>
<th>Conditions</th>
<th>O₂ Consumption (µmol·(g fresh wt)⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GL66</td>
</tr>
<tr>
<td>Control</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>+ KCN</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>+ SHAM</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>+ KCN and SHAM</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
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* Numbers in parentheses, SE.
slightly inhibited by KCN alone (Fig. 2, A and B). That is, although the mature tissue had substantial alternative path capacity, this path did not contribute to O₂ uptake in the absence of KCN (note that the lack of effect of KCN was not due to its poor penetration, because addition of KCN after SHAM inhibited O₂ uptake severely; Fig. 2, A and B). O₂ uptake by the leaf base slices, on the other hand, was severely inhibited by KCN alone (Fig. 2C). That is, the capacity of the alternative path in meristematic leaf tissue was much less than that in mature leaf tissue. Presumably, the alternative path capacity increases during leaf development.

**Regulation of Mature Leaf Respiration.** The manner in which leaf respiration in the two *Lolium* populations is regulated was investigated by the use of various effectors and substrates. It should be noted that, unlike wheat leaf respiration (3), *Lolium* leaf respiration showed no significant diurnal variation in the rate of respiration (not shown). In wheat leaves, respiration rates varied diurnally as a result of fluctuations in leaf sugar levels (1, 3). *Lolium* leaf sugar levels, on the other hand, did not change significantly during the night or even after 30 h of continuous darkness (Table II). Consequently, further experiments were carried out only on leaves harvested near the middle of the day.

Adding an uncoupler (CCCP) stimulated the rate of O₂ uptake by both lines by approximately 20% and subsequent addition of sucrose stimulated it further (Table IIIA). Addition of sucrose in the absence of CCCP, however, had virtually no effect on respiration (Table IIIB), although adding glycine alone stimulated it by 15–20% (Table IIIC). Addition of CCCP after glycine stimulated it further (Table IIIC). These results suggest that the rate of respiration in both lines was restricted by adenylate control (i.e. by high cytosolic energy charge) of glycolysis, which was relieved by adding uncoupler. The stimulation observed with glycine alone, which is metabolized directly by the mitochondria, shows that electron transport per se was not restricted.

This regulation via glycolysis was the same in both GL66 and GL72 (Table III) showing that the faster rates of O₂ uptake which characterize GL66 (Table I) are due simply to provision of more substrate to the mitochondria in the leaf cells.

Although sucrose plus CCCP, and glycine, stimulated O₂ uptake, subsequent addition of SHAM alone still did not inhibit respiration (Table IIID and E). That is, the alternative pathway remained disengaged even in the presence of exogenous substrates. KCN, however, inhibited respiration significantly in the presence of CCCP and sucrose (Table IIID), allowing an estimate of the capacity of the alternative path to be made. This capacity was the same percentage of total measured O₂ uptake in both GL66 and GL72, but was larger, in absolute terms, in GL66 (the faster respiring line). Since the respiration of both lines was regulated in the same manner, results of further experiments on
The rates of O₂ uptake in leaves from both Lolio populations are summarized in Table V. Interestingly, the respiration rate of the meristematic leaf bases was not stimulated by any combination of substrates or uncoupler (not shown) and in GL66, O₂ uptake by mature leaf slices in the presence of a cocktail of substrates and uncoupler were at least as fast as that by the meristematic tissue (Table V).

**Root Respiration.** Table VI summarizes measurements of O₂ uptake made with mature roots from both populations. Very little difference in the rate of O₂ uptake in either the presence or the absence of respiratory inhibitors was observed between GL66 and GL72. Respiration in the presence of KCN was substantial and the inhibition by SHAM alone indicates that the alternative path contributed approximately 35% to the total O₂ uptake. Addition of uncoupler stimulated O₂ uptake significantly in the presence of SHAM, suggesting that the Cyt path in these roots was restricted by ADP supply, with electrons spilling over into the alternative path. Addition of KCN and SHAM together inhibited respiration by at least 90%. The degree to which the alternative oxidase contributed to overall respiration was approximately the same for both lines. The ratio of the alternative oxidase activity to the Cyt oxidase activity was 0.60 for GL66 and 0.52 for GL72.

**Chemical Composition of Leaf Material.** There were no significant differences in the concentration of soluble sugars (glucose plus fructose) between the two populations (Table II). At the end of the night, and also after 30 h of darkness, the soluble sugar concentration was the same as that after several h of photosynthesis.

Both the soluble (‘amino acids’) and the insoluble (‘protein’) reduced N-concentrations were the same in both populations (Table II).

**Anatomical Studies.** The leaves showed the typical grass leaf anatomy, with ridges and grooves at the adaxial side. The vascular bundles in the ridge were surrounded by two bundle sheaths. The transverse distance between the bundles was constant and the same for leaves of both populations. Consequently, the wider leaves had more ridges and bundles than the narrower ones. In the grooves, the epidermal cells were enlarged, forming conspicuous bulliform cells. The mesophyll showed no differentiation into palisade and spongy parenchyma. It consisted of transverse plates, separated by more or less irregular intracellular spaces. Branched cells were numerous in the mesophyll.

The leaves of GL72 were thicker than those of GL66. The average thickness of GL72 was 0.28 mm (ridges) and 0.13 mm (grooves), versus 0.17 and 0.10 in GL66. Also, the vascular
bundled in GL72 were somewhat wider. The only distinct anatomical difference was the volume of intercellular space in the mesophyll. In longitudinal leaf sections, the amount of intercellular spaces was 47% (ridges) and 60% in GL72 versus 22 and 50% in GL66. Thus, in the thicker leaves of GL72 only the intercellular spaces were larger. The mass of the cellular material was approximately the same in both populations. Also, the specific leaf weight (g fresh weight per m²) was the same for leaves of the contrasting populations.

**DISCUSSION**

**Fast Respiring versus Slow Respiring Populations.** The results presented here confirm that the *L. perenne* GL66 population has higher rates of mature leaf respiration and slower growth compared to GL72. However, the respiration rate of meristematic leaf tissue, and the roots, is approximately the same in both. Thus, the increased biomass produced by GL72 reflects principally more efficient use of assimilated carbon in the fully expanded leaves. However, the similarity in the rate of root respiration also warrants further investigation: the faster growing roots are expected to respire faster.

The difference in dark respiration in mature leaves between the two lines is due to a difference in total respiratory capacity rather than a difference in the manner in which respiration is regulated. The lack of effect of added sucrose (alone) on O₂ uptake and the stimulation by uncoupler, glycine, and malate in both lines suggests that in the mature leaf, respiration in the dark is limited by substrate supply to the mitochondria. This limitation is brought about by adenylate control of glycolysis (the stimulation of O₂ uptake by glycine and malate in the absence of uncoupler, and the lack of effect of SHAM, an inhibitor of the alternative path, show that Cyt path activity per se was not limited).

Since SHAM had no effect on uncoupled respiration even when a cocktail of substrates was added (Table IV), it was not possible to estimate the full capacity of the Cyt chain (see "Materials and Methods"), but the maximum activity measured was greater in GL66 than in GL72 (Table V). This is also true for the alternative path, which did not contribute to O₂ uptake under the conditions imposed in our experiments.

Comparison of the O₂ uptake rate measured in the absence of effectors to the combined estimated capacities of the alternative and Cyt pathways (Table V) shows that in both lines only approximately one-third of the total respiratory capacity of the mitochondria was used in the dark. However, since the total capacity of GL66 is higher than that of GL72, O₂ uptake in the dark was faster in GL66. Whether the greater electron transport capacity of GL66 is due to higher specific mitochondrial activity or to more mitochondria per g fresh weight cannot be assessed yet. The similar effects of substrates and uncoupler on the respiration of the two lines shows that glycolysis limits O₂ uptake in both. Control of glycolysis presumably involves high cytosolic ATP:ADP ratios. Such high ratios have indeed been observed, using 31P nuclear magnetic resonance spectroscopy (11).

It therefore appears that the higher O₂ consumption by GL66 reflects faster turnover of cellular ATP. This in turn implies that energy consuming processes in mature leaf cells in the fast respiring population are less efficient than those in the slow one. Processes such as metabolite translocation and protein turnover are possibly involved here. The analysis of respiratory control outlined above also suggests that there are unlikely to be severe penalties incurred by selection for slow respiration in *Lolium*.

**Possible Causes of a Higher ATP Demand in the High Respiration Population.** The present results do not provide a definite clue to explain the increased ATP demand of GL66 plants. However, the similarity in chemical composition (Table II) and in anatomy indicate that the increased demand for ATP is due to neither an increased net rate of protein synthesis nor increased distances for the transport of sucrose from the chloroplast to the phloem.

**Regulation of Respiration in Different Tissues.** The stimulation of O₂ uptakes in mature leaf slices by malate, glycine, and uncoupler indicates that substrate presentation to the mitochondria limited overall respiration in this tissue and further indicates that different pathways are used for the metabolism of different substrates. Sucrose is metabolized via glycolysis, giving rise to pyruvate, while glycine and malate presumably enter the mitochondria directly to be oxidized, respectively, by glycine decarboxylase and NAD-malic enzyme, thus bypassing adenylate control of glycolysis. However, it should be noted that it is also possible that different substrates have their effects on different cell types, since the leaf is a heterogeneous tissue, although the bulk of O₂ uptake probably occurs in the more numerous photosynthetic cells.

The lack of saturation of the electron transport chains in the presence of several different substrates probably reflects restriction on substrate entry into the leaf slices or sequestration of some substrates by compartments other than the mitochondria. It is interesting to note that although endogenous sugar levels were quite high in these leaves, exogenous sucrose stimulated O₂ uptake when uncoupler was present. This suggests that not all of the endogenous sugars were available to respiration, implying control by compartmentation.

The control of respiration in *Lolium* leaves is very different from that found in wheat leaves. In the latter tissue, respiration rate is controlled in the first instance by the concentration of sugars in the leaf (3). When sugars are plentiful, however, control of the Cyt chain activity by ADP:ATP becomes evident in wheat leaves, with electrons from substrate oxidation spilling over into the alternative path (2). That is, in wheat leaves, substrate presentation to the mitochondria exceeds the activity of the Cyt chain which is restricted by adenylates. The reverse situation occurs in *Lolium* leaves. It is interesting to note that malate stimulates *Lolium* leaf respiration (Table III) but apparently not that of wheat leaves (J. Azcon-Bieto, personal communication). It is tempting to speculate that in wheat leaves malate is produced endogenously via phosphoenolpyruvate carboxylase and malate dehydrogenase in the cytosol during metabolism of sugars, bypassing adenylate control points of glycolysis and allowing expression of the alternative path (6). Clearly, a more detailed comparison of these tissues is warranted.

The pattern of respiration also varies from tissue to tissue within a given plant species. Respiration rates of the meristematic leaf base of *Lolium* were higher than those of the mature leaf (Table I) and did not respond to uncoupler or exogenous substrates, suggesting that respiratory flux was near maximal in this tissue. This presumably reflects greater demand for ATP in meristematic regions, an idea supported by the very low capacity of the alternative path in the leaf base (Table V). It should be noted that measurements were made on segments taken from within the leaf sheath and hence the tissue was probably not photosynthetic (it was a very pale yellow color). Thus, lack of effect of glycine here may have been due to the absence of glycine decarboxylase. Development of the alternative path in the upper portions of the leaf may have been a consequence of greening rather than maturation, since alternative path capacity in (green) bean and pea leaves did not change during leaf development (4).

Root respiration provided a more dramatic illustration of the flexibility of the respiratory control in plant tissues (Table VI). Here, SHAM alone inhibited respiration substantially, showing that the alternative path contributed to O₂ uptake, and subsequent addition of uncoupler relieved this inhibition. This indicates that root respiration was, at least in part, restricted by adenylate control of the Cyt path, with glycolytic flux not...
matched to the cell’s energy demand. This is in marked contrast to the leaf where ATP turnover strictly limits glycolysis (see above). Thus, care must be exercised when making generalizations about the nature of respiratory control in plants.

Finally, it is interesting to note that the respiratory capacity of mature leaves was not fully realized in the dark in our experiments. Similar observations have been made with other tissues (2–4). This may indicate that mitochondrial respiration is faster in the light than in the dark when supply of substrates may be greater (1). This in turn implies that the nature of respiratory control is different in the light from that in the dark.

Acknowledgments—We thank Dr. Rinie Hofstra for her critical comments on a draft of this manuscript and Mr. Colin J. Pilbeam for his assistance with the chemical analyses.

LITERATURE CITED
