Selection and Characterization of Tobacco Plants with Novel O₂-Resistant Photosynthesis

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ABSTRACT

Plants were obtained with novel O₂-resistant photosynthetic characteristics. At low CO₂ (250–350 µL CO₂ L⁻¹) and 30°C when O₂ was increased from 1% to 21% to 42%, the ratio of net CO₂ uptake in O₂-resistant whole plants or leaf discs compared to wild type increased progressively, and this was not related to stomatal opening. Dihaploid plantlets regenerated from anther culture were initially screened and selected for O₂-resistant growth in 42% O₂/160 µL CO₂ L⁻¹ and 0.18% of the plantlets showed O₂-resistant photosynthesis. About 30% of the progeny (6 of 19 plants) of the first selfing of a fertile plant derived from a resistant dihaploid plant had O₂-resistant photosynthesis, and after a second selfing this increased to 50% (6 of 12 plants). In 21% O₂ and low CO₂, net photosynthesis of the resistant plants was about 15% greater on a leaf area basis than wild type. Net photosynthesis was compared in leaf discs at 30 and 38°C in 21% O₂, and at the higher temperature O₂-resistant plants showed still greater photosynthesis than wild type. The results suggest that the O₂-resistant photosynthesis described here is associated with a decreased stoichiometry of CO₂ release under conditions of rapid photorespiration. This view was supported by the finding that leaves of O₂-resistant plants averaged 40% greater catalase activity than wild type.

Photosynthesis in C₃ plants is inhibited 33 to 55% by the 21% O₂ in the atmosphere compared with rates at 1 to 3% O₂. To call attention to the importance of this effect, I have referred to it as oxygen stress (28). Inhibition by 21% O₂ is completely reversed by high CO₂, showing that it is related to photorespiration. One obvious way of decreasing this adverse effect of O₂ in normal CO₂ would be to increase the CO₂/O₂ specificity of ribulose bisphosphate carboxylase/oxygenase (10).

In the oxidative photorespiratory pathway, two glycine molecules condense to produce one serine and one CO₂; hence, the stoichiometry should be 25% of the carbon flux if this is the sole source of photorespiratory CO₂ (26). However, recent experiments conducted at low CO₂ concentrations (5, 6) indicate that the stoichiometry of photorespiration in vivo can exceed 25% when the ratio of net photorespiration/net photosynthesis is elevated by high O₂ (above 21%) or higher temperatures. This suggests an alternative approach to the improvement of photosynthetic efficiency in C₃ plants based on identification of mutants that avoid this increase in stoichiometry of CO₂ release under conditions of rapid photorespiration.

Here it is demonstrated that atmospheres of 42% O₂/low CO₂ inhibit growth of tobacco seedlings, and that the inhibition is reversed by high CO₂. I have therefore screened a population of dihaploid tobacco plantlets for survival and growth in a 42% O₂/low CO₂ atmosphere and studied the characteristics of selected plants with O₂-resistant photosynthesis in populations of two generations of fertile plants derived from a dihaploid. These plants exhibit superior net photosynthesis under certain conditions, and their characteristics of O₂-resistant photosynthesis are consistent with their possessing a lower stoichiometry of photorespiratory CO₂ formation.

MATERIALS AND METHODS

Growth of Plants

Tobacco (Nicotiana tabacum, L. cv Havana Seed) that had undergone numerous self-pollinations without selection and further random selfings of this line were used as wild type. Seeds were surface-sterilized with dilute hypochlorite solution and germinated under continuous illumination (80 µmol photons m⁻² s⁻¹) at 27°C on RN agar medium (16). Seedlings were transplanted to small plastic pots containing a commercial mixture of peat-vermiculite-perlite, and were usually grown in a greenhouse set at a minimum temperature of 15°C and watered weekly with a complete nutrient solution.

Dihaploid plantlets were regenerated from anthers of immature flower buds cultured on agar medium (1, 11). They were transferred to polyurethane pads in Petri plates (15) containing liquid medium without sucrose and were grown in air for 1 week in light until they were green. Small (5 mm) plantlets were screened for O₂-resistant growth by transferring them to illuminated (100 µmol photons m⁻² s⁻¹) Plexiglas chambers that were flushed continuously for 2 weeks with moistened 42% O₂ containing sufficient CO₂ to maintain the concentration in the chamber at about 160 µL/L at 29°C. Under these conditions wild-type plantlets yellowed and some died, while a small number of O₂-resistant plantlets remained green and grew. Oxygen-resistant and O₂-sensitive plantlets from the same plate were recovered in 21% O₂/1% CO₂, transferred to rooting medium (11), and after further development were grown in the commercial potting mix in a controlled environment chamber (26°C, 22 h d at 100 µmol photons m⁻² s⁻¹) and then in the greenhouse. Net photosynthesis was determined on whole plants as described below.

To obtain fertile plants for pollinations, a number of stem cuttings were made of one of the O₂-resistant dihaploid plants,
42–12. These were grown in the greenhouse, and, as plants matured, lateral buds were painted with 0.6% colchicine in 1% gum tragacanth (3) to obtain axillary shoots with fertile flowers. One of the cuttings, designated 42–12F, produced large numbers of fertile flowers and its progeny were examined more extensively (Fig. 1). Randomly selected wild-type plants of the original parental line and progeny of further selfings of this line were used when photosynthetic and net assimilation rate comparisons were made with these selected plants.

To determine the mean net assimilation rate, seedlings growing in small pots were arranged according to size, and adjacent plants were paired. One plant of each pair served as the zero time control and its leaf area (Delta-T Area Meter) and total dry weight of tops and roots were determined (48 h at 85°C). The process was repeated with the second of each pair after about 7 d growth to make the individual comparisons. Mean net assimilation rates were determined from the relationship: 

\[ \frac{W_2 - W_1}{(t_2 - t_1)} \times (\log A_2 - \log A_1)/(t_2 - t_1) \]

where \( W \) is the total dry weight (mg), \( A \) is the leaf area (dm²), and \( t_2 - t_1 \) is the growth time, in days (21). To control concentrations of \( O_2 \) and \( CO_2 \), seedlings were grown in 50 L Plexiglas chambers that were continuously flushed at 1 L/min with moistened gas of known composition. The maximum leaf area per plant after 14 d growth was about 3 dm².

**Measurement of Photosynthesis**

Whole potted seedlings with four or five leaves were placed in a pair of 1 L Plexiglas chambers each containing a fan and illuminated from above at about 500 μmol photons m⁻² s⁻¹ (light saturation is usually observed at 600 to 700 μmol photons m⁻² s⁻¹) at about 30°C. Plants were flushed continuously with a stream of a moistened gas containing a fixed \( O_2 \) and \( CO_2 \) composition until steady state rates of photosynthesis were established. In some experiments, \( CO_2 \) gas exchange was determined by briefly closing off the chamber and measuring the \( CO_2 \) depletion rate by removing samples periodically with a hypodermic syringe and assaying their \( CO_2 \) content with an infrared \( CO_2 \) gas analyzer (20). The \( CO_2 \) concentration was adjusted so that the average level during the \( CO_2 \) depletion was about 350 μL/L. Five determinations of \( CO_2 \) assimilation were usually made before the flushing gas was changed and the steady state photosynthesis rate was determined again under the new conditions. The sequence followed when changing \( O_2 \) concentration was 21, 1, 42, and again 21% \( O_2 \). The second set of determinations at 21% \( O_2 \) established that the changes in photosynthesis were fully reversible. Gas mixtures of known composition were prepared by blending different proportions of \( CO_2 \)-free air, 1% \( CO_2 \)-air, \( N_2 \), and 43% \( O_2-N_2 \).

In most experiments the same Plexiglas chambers were used in a completely open system. The \( CO_2 \) concentrations in the entering and exiting gas streams were monitored by sampling with a hypodermic syringe and the flow rate entering the chamber was measured. Net photosynthesis was then determined from the difference in \( CO_2 \) concentration times the flow rate. Leaf areas of intact plants were obtained by determining length times width (cm) times 0.7.

Photosynthesis in tobacco leaf discs was determined by cutting 18 1.6-cm discs (0.36 dm² per leaf surface) with a sharp punch from expanding (5–12 g) leaves from the greenhouse. The discs were placed upside down in an open 8.5 cm Petri plate containing a thin layer (5 mL) of water. The discs were aligned in the Plexiglas chambers and flushed continuously with a moistened gas of known composition. The \( CO_2 \) gas exchange was measured after at least 90 min in the light (500 μmol photons m⁻² s⁻¹) using the open system at about 30°C. When stricter temperature control was required to study the effect of increased temperature on net photosynthesis, the discs were placed instead in a jacketed 90 mL Plexiglas chamber while temperature-controlled liquid was circulated around the chamber (19). The discs were illuminated from below (500 μmol photons m⁻² s⁻¹), the atmosphere in the chamber was stirred vigorously with a fan, and the leaf temperature was monitored with a thermistor probe touching the top of one of the leaf discs (OL-729 Thermistor, Omega Instruments, Stanford, CT). Leaf temperature was controlled within 0.1°C in this system. The \( CO_2 \) gas exchange was also measured in an open system when using this chamber with flow rates of 0.4 to 0.5 L/min.

**Enzyme Assays**

To assay catalase activity, a 0.5 g sample of leaf lamina was ground in a glass homogenizer with 5 mL cold K phosphate buffer (0.05 M, pH 7.5) containing 5 mg of DTT (8). The suspension was centrifuged for 15 min at 27,000g. The clear supernatant was assayed for catalase activity by measuring the linear rate of decrease in absorbancy of H₂O₂ at 240 nm. One unit is defined as the activity catalyzing the decomposition of 1 μmol H₂O₂ per min.

Glycolate oxidase activity was assayed from the initial rate of O₂ uptake (31). The reaction mixture (pH 9.0) contained excess catalase and flavin mononucleotide. One enzyme unit represents 1 μmol glycolate oxidized per min. Protein was determined colorimetrically using a Coomassie blue reagent (Bio-Rad Laboratories) with bovine serum albumin as a standard.
Stomatal Measurements

Stomatal widths were measured from silicone rubber impressions made on a pair of leaf discs taken after obtaining steady state photosynthesis at 250 μL CO₂ L⁻¹ at different O₂ concentrations (24). The viscous rubber suspension (RTV 11, Silicone Products Division, General Electric Co.) was mixed with sufficient catalyst (stannous octoate) and rapidly spread over the exposed leaf disc surface so that the rubber vulcanized within about 1 min. A 'positive' was then prepared from the impression after coating it with colorless nail polish diluted one-third with acetone. The film thus produced was fixed to a glass slide with a drop of water and 20 randomly selected stomatal widths were measured on each disc using a microscope at ×1000 magnification.

RESULTS

Screening System for O₂-Resistant Growth

Earlier results suggested that selecting plants for superior growth at elevated oxygen levels might provide a method of identifying selections of C₃ species with decreased photospiration and increased net photosynthesis (28) and that exposure to 60% O₂ for several weeks was lethal to tobacco seedlings or haploid plantlets (27). Elevated O₂ levels may produce toxicity through a number of mechanisms, but oxygen stress caused primarily by photospiration should be reversed at saturating CO₂ levels (1%). Net photosynthesis and growth should then be expected to be the same in high O₂/1% CO₂ as in 21% O₂/1% CO₂. Growth of tobacco seedlings was evaluated by determining the mean net assimilation rate in a controlled environment as a conservative measure of photosynthetic efficiency under different O₂ and CO₂ concentrations. I found that the toxic effects of 60% O₂/300 μL CO₂ L⁻¹ on the mean net assimilation rate were not reversed by 1% CO₂, indicating that some of the toxicity at 60% O₂ is caused by non-photospiratory mechanisms.

Similar experiments were therefore performed with tobacco seedlings using 42% O₂ and still lower concentrations of CO₂. Table I, experiment 1, shows that over a 7-d growth period 42% O₂/160 μL CO₂ L⁻¹ strongly decreased the mean net assimilation rate compared with 21% O₂/160 μL CO₂ L⁻¹. Increasing the CO₂ concentration to 1% completely reversed the inhibitory effect of 42% O₂ and restored growth rates to those at 21% O₂/1% CO₂. In experiment 2, the complete reversibility at 42% O₂ is further substantiated. Inhibition of growth for 7 d at 42% O₂/200 μL CO₂ L⁻¹ was fully reversed by 42% O₂/1% CO₂ even when the plants had been exposed to 42% O₂/200 μL CO₂ L⁻¹ for the first 7 d. Moreover, growth continued slowly in 42% O₂/200 μL CO₂ L⁻¹ and was similar for the first and second 7 d periods.

Thus, 42% O₂/low CO₂ was established as a suitable system for mass screening of dihaploid tobacco plantlets for resistance to oxygen stress. This system permitted large numbers of plantlets to be screened in a constant environment that could be easily monitored for O₂ and CO₂ concentration. Anther-derived dihaploid plantlets were employed in order to take advantage of the high rate of spontaneous mutations in plants regenerated from tissue culture (13). Of 2714 dihaploid plantlets grown for 2 weeks in an atmosphere of 42% O₂/160 μL CO₂ L⁻¹, 26 (0.96%) were classified as O₂-resistant based on growth and 5 of these (0.18%) showed O₂-resistant photosynthetic characteristics different from wild type (29).

Photosynthetic Characteristics of O₂-Resistant Dihaploids

The O₂-resistant plantlet most extensively studied was 42-12. The effect of 21, and 42% O₂ on net CO₂ exchange of this plantlet was compared with 42-13 (a control plantlet rescued from the same Petri plate) in three experiments conducted over a 13-d period (Table II). Net photosynthesis was similar in the two plantlets at 1% O₂, and was significantly higher in 42-12 than in wild type at 21% O₂ (+12.4%) and at 42% O₂ (+28.2%). The relation of net CO₂ uptake of the O₂-resistant plantlet to the wild type expressed as a ratio thus increased progressively with increasing O₂ level.

This novel characteristic of a steady relative increase in net photosynthesis on raising the O₂ level was used as the criterion in subsequent generations for identifying plants possessing O₂-resistant photosynthesis. It was shown by a progressive increase of 10% or more in the ratio of net photosynthesis of O₂ resistant/wild type at each step on going from 1 to 21 to 42% O₂.

Photosynthetic Characteristics of Progeny of a Doubled Dihaploid O₂-Resistant Plant

Fertile plants were required to study the biochemistry and genetics of the mechanisms of O₂-resistant photosynthesis. Treatment of plant 42-12F, derived from a stem cutting of
Table II. Effect of O₂ Concentration on Net Photosynthesis of Dihaploid Plantlet 42-12

The O₂-resistant (OR) and susceptible (WT) plantlets were initially recovered from the same Petri plate. They were then grown in air in a constant environment chamber, and net photosynthesis (PS) was determined in three separate experiments conducted over a 13 d period. The plants were flushed continuously with different concentrations of O₂ containing about 350 µL CO₂ L⁻¹, at 300 µmol photons m⁻² s⁻¹ and 30°C. The measurements were made in the sequence 21% O₂, 1% O₂, 42% O₂, and again 21% O₂, and all values at 21% O₂ were used to obtain the means (±SD). Total leaf areas varied from 1.4 to 3.2 dm². A two-way analysis of variance was conducted, n=12 for 1% O₂; n=24 for 21% O₂; and n=12 for 42% O₂; the least significant difference of the means (LSD) are shown.

<table>
<thead>
<tr>
<th>O₂ Concentration</th>
<th>Mean Net Photosynthesis</th>
<th>LSD, P&lt;0.01</th>
<th>Significant Difference of Means</th>
<th>Net PS OR/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂-resistant plantlet 42-12</td>
<td>Wild-type plantlet 42-13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>mg CO₂ dm⁻² h⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16.1</td>
<td>15.2</td>
<td>2.55</td>
<td>NS</td>
</tr>
<tr>
<td>21</td>
<td>10.8</td>
<td>9.61</td>
<td>0.95</td>
<td>0.01</td>
</tr>
<tr>
<td>42</td>
<td>7.81</td>
<td>6.09</td>
<td>0.78</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table III. O₂-Resistant Photosynthesis in Seedlings Derived from Selfing Colchicine-Treated Dihaploid Plant 42-12F

Photosynthesis (PS) was measured in randomly selected intact wild-type (WT) and potentially O₂-resistant (OR) seedlings at 550 µmol photons m⁻² s⁻¹. The total leaf area of the seedlings varied from 0.4 to 1.8 dm². Mean net photosynthesis values were obtained from 4 to 6 measurements under steady state conditions. Capital letters identify individual sibling plants with O₂-resistant photosynthesis. Values shown are means of two experiments.

<table>
<thead>
<tr>
<th>OR Plant</th>
<th>Inhibition of PS, 42% O₂ vs. 21% O₂</th>
<th>Net PS Ratio OR/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>1% O₂</td>
</tr>
<tr>
<td>F</td>
<td>38</td>
<td>51</td>
</tr>
<tr>
<td>I</td>
<td>48</td>
<td>54</td>
</tr>
</tbody>
</table>

42-12 (Fig. 1), with colchicine produced fertile flowers, and on self-pollination the progeny of this first selfing were compared with wild-type plants for O₂-resistant photosynthesis. The characteristics of sibling plants F and I are shown in Table III. Six of 19 intact plants that were derived from plant 42-12F consistently showed O₂-resistant photosynthesis when compared with wild type at 1, 21, and 42% O₂. Thus, about 30% of this population had photosynthetic properties distinct from wild type with respect to the inhibition of photosynthesis by oxygen.

Photosynthetic Characteristics of Progeny of Second Self-Pollination of an O₂-Resistant Plant

One randomly selected wild-type plant and O₂-resistant selection I (Table III; Fig. 1) were selfed and their progeny were examined for O₂-resistant photosynthesis. In this generation, 6 of 12 plants studied were identified as O₂-resistant. The combined photosynthesis rates of three of these plants from the same planting are compared with three randomly selected wild-type plants with which they were paired in Table IV. Relative to wild type, photosynthetic rates were the same in 1% O₂, while a significant increase occurred in the O₂-resistant plants in 21% O₂ (+16.5%) and in 42% O₂ (+36.4%). Thus, in progeny of the second selfing, about 50% of the plants exhibited O₂-resistant photosynthesis compared to about 30% in the previous generation.

For biochemical studies it would be useful to be able to conduct photosynthetic experiments with leaf discs. The photosynthetic characteristics of leaf discs of two plants from the second selfing (Table IV) were therefore examined and their O₂-resistance was confirmed (Table V). Net photosynthesis was significantly greater than in wild type at 42% O₂ whether or not CO₂ assimilation differed from wild type at 1% O₂.

The effect of CO₂ concentration on net photosynthesis of leaf discs was examined on wild type and O₂-resistant leaves using an open system in 21% O₂ at about 30°C. At 250 µL CO₂ L⁻¹ net CO₂ exchange (mg CO₂ dm⁻² h⁻¹ for one leaf surface) was 14.0 for wild type and 16.6 for the O₂-resistant plant (+19%); at 340 µL CO₂ L⁻¹ the values were, respectively, 20.8 and 22.8 (+9.6%); at 540 µL CO₂ L⁻¹ similar rates were obtained in both types, 32.2; and at 1300 µL CO₂ L⁻¹ similar rates were again observed in both types, 47.7. Thus, the responses to CO₂ concentration for wild-type and O₂-resistant leaf discs were normal and similar, except that photosynthetic rates were greater for the O₂-resistant leaf at lower CO₂ concentrations.

Catalase and Glycolate Oxidase Activities of Plants with O₂-Resistant Photosynthesis

Catalase activities were assayed on 11 different days during a 1 month period in leaf extracts from three O₂-resistant plants (Table IV) and wild-type plants growing in the greenhouse. Table VI, experiment 1, summarizes results that show catalase activity in O₂-resistant plants exceeded wild type in all 11 comparisons. It should be noted that the specific activities for catalase in wild type are high for this species (8). The mean catalase activity in the O₂-resistant plants was a highly significant 40% greater. Experiment 2, carried out about 10 weeks later on two of the same O₂-resistant plants, shows that catalase activity on a protein basis was always greater while glycolate oxidase activity did not differ from wild type.

Effect of O₂ and Stomatal Opening on Net Photosynthesis in O₂-Resistant Plants

Table VII compares net photosynthesis in 42% O₂ in leaf discs of three O₂-resistant plants with three different wild-type plants from a later planting. The characteristic large increase in net photosynthesis is shown in O₂-resistant discs at 42% O₂. Stomatal widths in the resistant plants were the same or narrower than wild type in 42% O₂, and also in 21% O₂ stomatal widths were not greater in O₂-resistant leaves than in wild type. Stomatal numbers per unit leaf area were the same in both kinds of leaves. In these experiments a higher catalase activity, average increase of 39% on a leaf area basis,
Table IV. O₂-Resistant Photosynthesis in Seedling Progeny of Second Selfing of an O₂-Resistant Selection from 42-12F

Photosynthesis (PS) was measured at 300 μL L⁻¹ in an open system in intact greenhouse-grown seedlings obtained after selfing plant I (Table III). Total leaf area of the seedlings varied from 0.6 to 1.3 dm². An analysis of variance was conducted of mean net photosynthesis values of three O₂-resistant (OR) plants (B, C, and D) against three randomly selected wild-type (WT) plants. At 1% O₂ n=13; 21% O₂ n=12; 42% O₂ n=12.

<table>
<thead>
<tr>
<th></th>
<th>OR plants B, C, D combined</th>
<th>WT plants combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>mg CO₂ dm⁻² h⁻¹</td>
<td>mg CO₂ dm⁻² h⁻¹</td>
</tr>
<tr>
<td>1% O₂</td>
<td>22.3</td>
<td>22.4</td>
</tr>
<tr>
<td>21% O₂</td>
<td>12.0*</td>
<td>10.3</td>
</tr>
<tr>
<td>42% O₂</td>
<td>6.15*</td>
<td>4.51</td>
</tr>
</tbody>
</table>

* P<0.05.

Table V. Demonstration of O₂-Resistant Photosynthesis in Leaf Discs of O₂-Resistant Plants from the Second Selfing of 42-12F

Eighteen 1.6 cm leaf discs, 0.36 dm², were cut from leaves (5–9 g fresh weight) of two different O₂-resistant (OR) and wild-type (WT) plants growing in the greenhouse. Net photosynthesis (PS) was measured in 500 μmol photons m⁻² s⁻¹ in an open system after the chamber was continuously flushed for at least 1.5 h with 21% O₂/250 μL CO₂ L⁻¹ (±sd).

<table>
<thead>
<tr>
<th>O₂ Concentration</th>
<th>Mean Net Photosynthesis</th>
<th>Net PS Ratio OR/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR plants</td>
<td>WT plants</td>
</tr>
<tr>
<td>%</td>
<td>mg CO₂ dm⁻² h⁻¹</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30.5±0.6**</td>
<td>27.4±0.2</td>
</tr>
<tr>
<td>21</td>
<td>16.6±0.2**</td>
<td>14.0±0.3</td>
</tr>
<tr>
<td>42</td>
<td>8.77±0.7**</td>
<td>6.57±0.2</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>29.5±0.2**</td>
<td>32.3±1.0</td>
</tr>
<tr>
<td>21</td>
<td>16.3±0.2</td>
<td>16.5±0.3</td>
</tr>
<tr>
<td>42</td>
<td>8.52±0.5*</td>
<td>7.38±0.4</td>
</tr>
</tbody>
</table>

* P<0.05. ** P<0.01.

was again confirmed in O₂-resistant plants compared to wild type.

Effect of Temperature on Net Photosynthesis of O₂-Resistant Plants

Increasing O₂ concentration or temperature increases the stoichiometry of CO₂ during photorespiration, as well as the ratio of photorepiration to net photosynthesis (5, 6). To test whether O₂-resistant photosynthesis was associated with enhanced photosynthesis at higher temperatures, the effect of higher leaf temperatures on CO₂ exchange of leaf discs floated on water was studied. The experiments were carried out using a system that permitted careful control of leaf temperature ("Materials and Methods"). Three different O₂-resistant plants from the second self-pollination (Fig. 1) were compared in these studies. In wild-type leaf discs, increasing leaf temperature from 30 to 38°C decreased net CO₂ assimilation about 20%, while in O₂-resistant plants the decrease was only about 10% (Table VIII). At the higher temperature, net photosynthesis was always significantly greater in O₂-resistant relative to wild-type plants. A three-way analysis of variance of net photosynthesis in O₂-resistant and wild-type plants against temperature was conducted for the three experiments in Table VIII, and the effect of plant type was highly significant (P < 0.001).

Experiments were carried out to determine whether the CO₂ compensation points differed between wild-type and O₂-resistant leaf discs, but the standard deviations of the determinations were too great to detect significant differences. The CO₂ compensation point values at 30°C were about 68 μL CO₂ L⁻¹ and at 38°C approximately 121 μL CO₂ L⁻¹. When the discs were flushed with CO₂-free air (0.45 L min⁻¹) at 38°C, wild-type discs released CO₂ in light at a mean rate equal to 28.2% of net CO₂ uptake compared with a value of 24.8% for O₂-resistant leaves. Although the differences were significant, they were small and are considered preliminary because few measurements were made under a single set of conditions.

DISCUSSION

Other examples of a decreased O₂-inhibition of CO₂ uptake in C₃ plants have been reported but the properties are distinctly different from those described here. For example, lower O₂-inhibition of photosynthesis has been observed among different cultivars of peas and beans (18) and the C₃-C₄ intermediate species may show a lower O₂-inhibition of photosynthesis than their C₃ counterparts (2, 17). However, net photosynthesis per unit leaf area in the intermediate species was not greater than in C₃ plants in normal CO₂ at 21% O₂. In the present study numerous measurements of net photosynthesis at 30°C and low CO₂ showed that in 21% O₂ the novel plants average about 15% greater net photosynthesis per unit leaf area than wild type.

Decreased O₂-inhibition of photosynthesis has also been observed under conditions associated with low concentrations of stromal phosphate (7), high light and CO₂ (22), and low temperature (14). The sequestering of phosphate decreased the capacity of 2% O₂ to stimulate photosynthesis and lowered the CO₂ concentration needed for saturation (7), but the O₂-resistant plants described here are saturated at CO₂ concen-
Table VI. Catalase and Glycolate Oxidase Activities in Leaves of Wild-Type and O$_2$-Resistant Plants from the Second Sowing

In experiment 1, leaves (5–8 g fresh weight) of five different randomly selected wild-type plants (WT) and the O$_2$-resistant selections (OR) were taken from the greenhouse over a period of 1 month, and samples were extracted and assayed for catalase activity as described under "Materials and Methods." Pairs of leaves were assayed in a random sequence on 11 different days. In an experiment in which the OR/WT ratio on a fresh weight basis was 1.79, catalase activity was 431 for wild-type and 749 for O$_2$-resistant (units/mg protein) giving a ratio of 1.74. In experiment 2, leaves (9–12 g fresh weight) of O$_2$-resistant plants C and D and wild-type were assayed about 10 weeks later. The number of determinations is shown in parentheses. An analysis of variance was conducted for all catalase assays on O$_2$-resistant and wild-type plants in experiments 1 and 2, and for glycolate oxidase activities in experiment 2.

<table>
<thead>
<tr>
<th>Mean Catalase Activity</th>
<th>Catalase Activity</th>
<th>OR/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$-resistant plants B, C, and D</td>
<td>units/g fr wt</td>
<td>units/g fr wt</td>
</tr>
<tr>
<td>6,940 (4)</td>
<td>4,990 (4)</td>
<td>1.40</td>
</tr>
<tr>
<td>9,460 (5)</td>
<td>6,380 (5)</td>
<td>1.48</td>
</tr>
<tr>
<td>6,760 (2)</td>
<td>6,390 (2)</td>
<td>1.06</td>
</tr>
<tr>
<td>Individual Totals</td>
<td>8,050**</td>
<td>5,880</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean Catalase Activity</th>
<th>Mean Glycolate Oxidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$-resistant plants C and D</td>
<td>units/mg Protein</td>
</tr>
<tr>
<td>Plant C</td>
<td>240 (2)</td>
</tr>
<tr>
<td>Plant D</td>
<td>222 (3)</td>
</tr>
<tr>
<td>Individual Totals</td>
<td>229**</td>
</tr>
</tbody>
</table>

** P<0.01.

piratory pathway (5, 6) or (c) diminishing the quantity of glycolate synthesized by other possible precursors besides ribulose bisphosphate (30). The quantitative contributions of other pathways of glycolate synthesis have not yet been established, so I will turn to the other two possibilities. However, still another explanation for O$_2$-resistant photosynthesis might be that leaf stomata open wider in such plants, especially in higher O$_2$ levels compared to wild type. This hypothesis is not borne out by the results in Table VII which show that stomatal widths in wild-type leaves are the same or wider than O$_2$-resistant at 42% O$_2$.

The oxygenase activity resides on the large subunit of the enzyme coded by chloroplast DNA that is present in multiple copies per cell. Extensive attempts to recover low oxygenase mutants of Arabidopsis have failed (23), presumably because of inherent difficulties associated with selection of induced chloroplast mutations at the whole plant level (16). It is therefore equally unlikely that my selection scheme based on O$_2$-resistant growth of dihaploid plantlets would recover mutants with decreased oxygenase activity.

Hanson and Peterson (5, 6) have described a direct attempt to determine the stoichiometry of the photorespiratory pathway in vivo under conditions removed from the CO$_2$ compensation point. They determined net photosynthesis, the CO$_2$ photorespired (using a sensitive method based on the postillumination transient), and simultaneously employed a sterchemical probe that responds primarily to the partitioning of ribulose bisphosphate between oxygenation and carboxylation. The stoichiometry calculated from these combined measurements was close to the 'expected' value at 21% O$_2$/340 μL CO$_2$ L$^{-1}$ and 25°C, i.e. about 25% of the flux of...
glycolate carbon was released as CO$_2$. Factors that increased the rate of photorespiration relative to net photosynthesis, such as O$_2$ concentration and temperature, increased the calculated stoichiometry to values well above 25%. The trend was illustrated, for example, when at 32°C in 21% O$_2$/340 $\mu$L CO$_2$ L$^{-1}$, the stoichiometry was 38%, while in 42% O$_2$ the stoichiometry increased to 55% (6). Thus, it seemed reasonable to suspect that plants selected for O$_2$-resistant growth might have O$_2$-resistant photosynthesis because the stoichiometry of photorespiration is decreased to a value closer to 25% even when the photorespiration/net photosynthesis ratio is high.

The photorespiratory metabolites glyoxylate and hydroxyacetooisocitrate are rapidly decarboxylated by H$_2$O$_2$, (4, 25, 31) and H$_2$O$_2$ is generated in the pathway by glycolate oxidase (31). Catalase is located in peroxisomes in close proximity to glycolate oxidase, and a barley mutantgrossly deficient in catalase was unable to survive under photorespiratory conditions but grew to maturity at saturating CO$_2$ concentrations (12). When photorespiration increases greatly, however, as at high levels of O$_2$ and higher temperatures, the excess H$_2$O$_2$ produced might successfully attack the vulnerable keto-acids of the pathway and generate CO$_2$. A plant could therefore be O$_2$-resistant because it possesses greater catalase activity (6) and show superior net photosynthesis at higher temperatures because less CO$_2$ is produced internally. Catalase is an enzyme whose activity is rapidly regulated by changing CO$_2$ concentra-

trations (8, 9) and plants with higher activities might be selected by screening for better growth in high O$_2$/low CO$_2$ as was done initially.

The O$_2$-resistant plants obtained from the second selfing averaged about 40% greater catalase activity on a fresh weight, protein, or leaf area basis than wild type (Table VI and “Results”). Enhanced catalase activity persists at all stages of leaf development examined thus far. In contrast, the activity of glycolate oxidase, another peroxisomal enzyme, did not differ from wild type (Table VI). The net photosynthetic rate in O$_2$-resistant leaf discs also increased significantly relative to wild type at 38°C compared to 30°C (Table VIII). Thus, the novel O$_2$-resistant phenotype of these plants may result from lower stoichiometry of CO$_2$ release under conditions of rapid photorespiration.

The progeny of a dihaploid plant with O$_2$-resistant photosynthesis, 42–12F (Fig. 1), showed 6 of 19 plants examined, about 30%, with this characteristic (Table III; and “Results”). On subsequent selfing of a plant with O$_2$-resistant photosynthesis, the frequency of resistant progeny increased to 6 of 12 plants tested, 50% (Table IV; and “Results”). This suggests that plants displaying the O$_2$-resistant phenotype that is correlated with high catalase activity are heterozygous for the mutant gene(s), resulting in segregation upon self-pollination. Continued selection of resistant plants for selfing should gradually increase the frequency of the mutant gene(s) and eventually produce homozygous mutants.

Further attempts to establish a causal connection between O$_2$ resistance and high catalase will be based on cosegregation of these traits in selfed progeny of O$_2$-resistant plants. A third selfing has been carried out and reciprocal crosses have been made with wild type to establish the relationships of these traits further. Analysis of the characteristics of this third selfing may help provide answers to some of the genetic and biochemical questions remaining about the mechanisms governing O$_2$-resistant photosynthesis of the kind described here.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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