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Regulation of Photosynthetic Rate of Two Sunflower Hybrids under Water Stress

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ABSTRACT

The effect of short-term water stress on photosynthesis of two sunflower hybrids (*Helianthus annuus* L. cv Sungro-380 and cv SH-3622), differing in productivity under field conditions, was measured. The rate of CO_2 assimilation of young, mature leaves of SH-3622 under well-watered conditions was approximately 30% greater than that of Sungro-380 in bright light and elevated CO_2; the carboxylation efficiency was also larger. Growth at large photon flux increased assimilation rates of both hybrids. The changes in leaf composition, including cell numbers and sizes, chlorophyll content, and amounts of total soluble and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein, and in Rubisco activity and amount of ribulose-1,5-bisphosphate (RuBP) were determined to assess the factors regulating the differences in assimilation of the hybrids at high and low water potentials. The amounts of chlorophyll, soluble protein, Rubisco protein and the initial activity of Rubisco and its activation state did not differ significantly between hybrids. However, unstressed leaves of SH-3622 had more, smaller cells per unit area and 60% more RuBP per unit leaf area than that of Sungro-380. Water stress developing over 4 days decreased the assimilation of both hybrids similarly. Changes in the amounts of chlorophyll, soluble and Rubisco protein, and Rubisco activity and activation state were small and were not sufficient to explain the decrease in photosynthesis; neither was decreased stomatal conductance (or stomatal "patchiness"). Reduction of photosynthesis per unit leaf area from 25 to 5 micromoles CO_2 per square meter per second in both hybrids was caused by a decrease in the amount of RuBP from approximately 130 to 40 micromoles per square meter in SH-3622 and from 80 to 40 micromoles per square meter in Sungro. Differences between hybrids and their response to water stress is discussed in relation to control of RuBP regeneration.

Photosynthetic CO_2 assimilation per unit area of leaf surface depends on the capacity of the plant's photosynthetic mechanism and on those environmental factors, such as CO_2 supply and radiation, which are the substrates for the process, and on those conditions (*e.g.* temperature and water supply) that affect the mechanism. The rate of photosynthesis, in combination with the leaf area, determines plant productivity (2, 10, 11, 18). Plants differ in their genetic potential for assimilation, *e.g.* C4 plants generally have a greater rate of CO_2 fixation than C3 plants in bright light and high temperatures, and the biochemical and physiological origins of this are well established (19). However, although there are differences in assimilation rate between species within the C3 and C4 groups, differences between closely related plants are less well established. Also, the causes of differences and how they relate to the characteristics of the photosynthetic mechanism between species and varieties are poorly understood (19, 21, 28).

Genetic variation in rates of photosynthesis per unit leaf area (Pn[2]) has been reported for cultivars of some crop species (2, 9, 12). Possible causes of the differences in Pn between genotypes are variations in amounts or activities of specific proteins, pigments, etc. and differences in leaf structure, cell size, or stomatal frequency. Small but consistent differences in assimilation were detected between hexaploid wheats under well-watered field conditions (9), but such differences between other wheat genotypes were attributed to ploidy (2). Substantial differences (up to 35%) have been reported between cultivars of field bean (13) and in pea (21), the latter attributed to variation in Chl content. Assimilation rates were best correlated with the ratio of cell surface area to leaf surface area (19), in analyses of 112 C3 and 6 C4 species. The correlations among cell size, ploidy, and assimilation rates (22) and also the effects of nutrition on the relationship (18) makes the analysis complex. Variation in Pn has also been attributed to the amounts and activities of Rubisco. Both the amount and the activity of Rubisco can limit photosynthesis in C3 plants (11, 18, 22, 26, 28, 29). Genetic variability in Rubisco content related to ploidy has been demonstrated (22), as has specific activity of the protein between C3 and C4 plants, although less within the groups (23). However, there is uncertainty about the importance of RuBP regeneration compared to Rubisco amount and activity (28, 29).

Assimilation also varies in response to environment. In this respect, productivity of plants is greatly decreased by water deficiency in most parts of the world and much emphasis is given to improving the drought tolerance of varieties (2). However, the effect of water stress on the mechanisms of CO_2 assimilation (3–5, 16, 19, 25) has not been analyzed in sufficient detail, in genotypes differing in assimilation capacity, to

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[2] Abbreviations: Pn, net photosynthesis; RuBP, ribulose-1,5-bisphosphate; Sungro, Sungro-380; SH, SH-3622; Ci, leaf internal CO_2 partial pressure; ψ, water potential(s); P_{max}, maximal rate of Pn; g_s, stomatal conductance; tsp, total soluble protein.

show how the photosynthetic mechanism may be modified to improve assimilation under water stress (9, 16, 21, 25, 26). The main limitation to assimilation under water stress is suggested as inhibition of photophosphorylation which decreases RuBP regeneration rather than the enzymatic or light reactions (4, 5, 16), but varietal responses have not been examined. Other studies (7, 25) suggest that the diffusion resistance offered to CO_2 by closure and "patchiness" of stomata is responsible for the decrease in photosynthesis caused by water stress.

In this paper we report differences in the maximum rates of Pn between sunflower (*Helianthus annuus* L.) cv SH and cv Sungro, with contrasted dry matter production under field conditions in southern Spain (C. Gimenez and E. Fererres, unpublished observations). The causes of the differences are related to cell numbers and sizes, to content of pigments and proteins, particularly Rubisco amounts and activity, and to RuBP content. Changes in Pn caused by short-term water deficits in the two genotypes are related to these features of metabolism and to cell composition.

MATERIALS AND METHODS

Two series of experiments were done in controlled environments. In one, photosynthetic rates of the two sunflower (*Helianthus annuus* L.) cultivars, Sungro and SH, were examined at three different levels of water stress in relation to differences in leaf structure and composition. In the second, the relation of Pn to the amount of RuBP and to the amounts and activities of Rubisco for the two genotypes under stress conditions was investigated.

Plant Growth

Plants were grown in plastic pots containing 10 L of F1 commercial compost containing all required nutrients in amounts sufficient to allow maximum growth. For the first experiment, the plants grew in a greenhouse (day temperatures between 20 and 25°C) in bright natural light during June. They were transferred to a controlled environment 1 week before water deficits were imposed. For the second experiment (in October), plants were grown in a warm glasshouse under small photon fluxes until the first pair of true leaves had unfolded before transfer to a controlled environment. Controlled conditions for both experiments were 25°C day, 15°C night temperatures and a PAR (400–700 nm) flux of 450 μmol quanta m^{-2} s^{-1} for 16 h, which is equivalent to the mean integral PAR of the United Kingdom in summer. Plants were watered twice daily and supplied weekly with 200 cm^3 of basal Hoagland solution with 4 mmol NO_3^-.

Three different watering treatments were imposed during 4 d before measurements by replacing 40, 70, or 100% of the water lost by evaporation. Preliminary experiments showed that these gave the required, approximately constant, leaf water ψs during the measurements.

Measurements of CO_2 and H_2O Exchange

Rates of Pn of both sunflower hybrids were measured on the fifth and sixth leaves within 1 week of full expansion. Measurements were made with chambers placed on 5.7 cm^2 of the lamina approximately 5 cm from the tip and 2 cm from the margin using a six-chamber open-circuit gas exchange with automatic data collection and analysis (18). Chambers had forced ventilation, and the temperatures were regulated to maintain the leaves at 20 ± 1°C (8). Plants were illuminated from a quartz-halogen lamp with 1200 μmol PAR m^{-2} s^{-1} at 20°C during measurements of the response of Pn to the Ci (14) varied in steps over the range 0 to approximately 80 Pa. To measure the response of Pn to radiation, the leaf was illuminated with 1500 μmol PAR m^{-2} s^{-1} for 10 to 15 min in air containing 34 Pa CO_2, 21 kPa O_2, and 79 kPa N_2 until assimilation was constant; radiation was decreased in steps by inserting neutral density wire screens between the light and chamber until the leaves were in darkness. The g_s and substomatal Ci were calculated according to methods described in ref. 8. Carboxylation efficiency of the leaves was calculated from the intial slope of the Pn/Ci relationship.

ψ and Osmotic Potential Measurements

Leaf ψ was measured using a pressure chamber (24) on the leaf opposite that used for gas exchange measurements and covered with plastic film immediately before detachment to decrease errors caused by water loss. Preliminary studies showed no significant differences between leaves. Osmotic potential was measured on frozen and thawed leaf samples using a vapor pressure osmometer (Wescor, Logan, UT).

Chl and Cell Number and Size Determinations

After measurement of Pn, three samples (approximately 2 cm^2) were removed from areas of the leaf used in the gas exchange studies from comparable positions and of similar appearance for the measurement of Chl, tsp, and cell numbers and sizes. Chl was analyzed in 80% (v/v) aqueous acetone extracts (1). Cell numbers were counted using a Coulter Counter (Coulter Electronics Ltd., Luton, U.K.) after separation of the cells with chromium trioxide (18). Cell volume was calculated from the fresh mass of the tissue and the cell number.

Determination of Protein and Rubisco Amount and Activity

Tsp was extracted in Bicine buffer (50 mM Bicine, 20 mM $MgCl_2$, pH 7.8) and determined (6) with BSA as standard. The amount of Rubisco protein was measured by PAGE of the native protein and identified and quantitated by comparison with standard sunflower and wheat Rubisco protein by staining with Coomassie brilliant blue in isopropanol-acetic acid-water, removing the bands followed by extracting in 1% SDS and spectroscopic determination (18, 23).

The activity of Rubisco was measured on the areas of leaf used to measure photosynthesis. When Pn was constant at the maximal rate at the end of the determination of the CO_2 response, the leaf area in the chamber was frozen to −20°C within 0.1 s by freeze clamping (17, 25). The Rubisco activity was determined by measuring the incorporation of $^{14}CO_2$ into acid-insoluble material as described in ref. 20 and modified

as described in ref. 18. Briefly, the leaf was ground to a powder in liquid nitrogen, followed by further grinding in buffer until thawed for an additional 30 s. Aliquots of the extract were assayed in duplicate at 25°C in a stirred oxygen electrode chamber (Hansatech Ltd., King's Lynn, U.K.), and $^{14}CO_2$ incorporation was allowed to proceed for 30 s, after which the reaction was stopped by addition of formic acid. The assay mixture was dried overnight at 80°C and ^{14}C determined by scintillation counting (Kontron, Münchenstein, Switzerland). The activation of the enzymes in the crude extract was allowed to proceed for 3 min at 25°C in the assay mixture, and the reaction was started by the addition of RuBP substrate. The reaction was allowed to continue for 30 s before addition of acid. The amount of RuBP was measured on the same tissue used for Pn determinations after freeze clamping and extraction in 2 cm^3 of 5% (v/v) perchloric acid, by measuring incorporation of $^{14}CO_2$ from the assay medium into acid-insoluble material. The action was catalyzed by activated Rubisco using the assay medium used for the enzyme activity but without RuBP (20).

Measurement of CO_2 Fluxes over Leaf Surface

The variation in the flux of CO_2 over the area of the leaf was determined by measuring the distribution of $^{14}CO_2$ across the area of leaf used for measurements of assimilation and, thus, to assess the variation in stomatal opening ("stomatal patchiness"). Leaves similar to those used for measurements of photosynthesis and biochemical components were used. Their photosynthesis was measured in 38 Pa CO_2, 21 kPa O_2, and 1200 μmol PAR m^{-2} s^{-1} in the freeze clamp system. When steady-state rates were achieved, g_s and Pn were measured, and immediately gas containing 38 Pa CO_2 (specific radioactivity 10.1 kBq μmol^{-1}) CO_2 was passed over the leaf for 30 s. Leaves were immediately freeze clamped and the pieces transferred, while fully frozen, to X-ray film (Fuji Film, London, U.K.) and clamped firmly to ensure good contact for autoradioagraphy. Exposure was at −18°C in darkness for approximately 2 weeks. The autoradiographs were scanned with a Joyce-Loebel scanning spectrophotometer at 80 μm resolution over several tracks the length of the freeze-clamped sample to determine the variation in film density. The total ^{14}C uptake by the leaf was obtained by combustion of leaf samples (Tricarb oxidiser 306, Packard, Caversham, U.K.). A report of the method and its application is in preparation.

Observations of the distribution of stomatal aperture over the leaf surface was also made using a solvent infiltration method (27) that integrates the effects of differences in stomatal characteristics (primarily aperture) over small areas of the leaf. Leaf discs (1 cm diameter) were punched from the leaf under similar light and CO_2 conditions used to measure gas exchange. The discs were agitated for 3 s in solutions of (a) 95% ethanol with waxolin blue dye (least penetrating), (b) absolute ethanol, (c) 4 volumes of absolute ethanol plus 1 volume of petrol ether, (d) 1 volume of petrol ether and 1 volume of absolute ethanol, (e) two volumes of petrol ether and 1 volume of ethanol (most penetrating). Solutions b to e contained cotton blue dye as indicator. The least penetrating solution that entered the leaf indicated the stomatal aperture. Closure of stomata in parts of the leaf was shown by reduction in the area infiltrated by a solution and irregular infiltration.

RESULTS

Sunflower Hybrids: Response of Photosynthesis to Ci, Radiation, and Water Stress

The rate of Pn as a function of the calculated internal Ci for each sunflower hybrid at low and high leaf ψ is given in Figure 1a. Under well-watered conditions ($\psi \simeq -0.6$ MPa), the Pn of SH was not statistically significantly different from that of Sungro between 0 and 20 Pa CO_2. However, the CO_2 saturated rate (at approximately 60 Pa CO_2) was about 30% higher in SH than in Sungro and remained so with increasing leaf age despite a substantial decrease in Pn until the leaves started to senesce. Maximum rates of Pn at CO_2 saturation decreased from the values indicated in Figure 1a, 37 d after sowing, to 17 and 21 μmol m^{-2} s^{-1} in Sungro and SH,

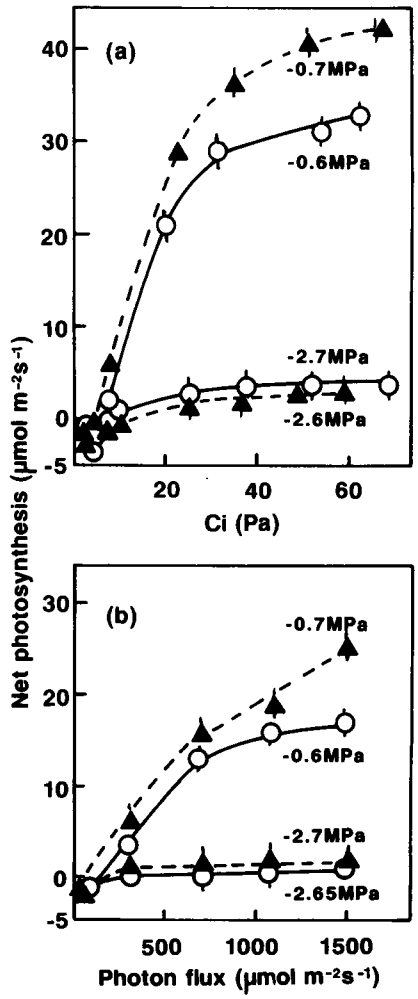

Figure 1. The response of Pn of two sunflower hybrids SH (▲) and Sungro (○) at different leaf ψ. a, Response to the leaf internal Ci at saturating photon flux. b, Response to photon flux at 36 Pa CO_2. Bars on points are ± SE of three replicates; values against curves are mean leaf ψ for each hybrid and stress interaction.

respectively, 55 d after sowing. The Pn of severely stressed leaves of Sungro and SH (at −2.7 MPa) were 12 and 10% of the control values; the varietal differences were not significant.

The response of the hybrids to radiation is shown in Figure 1b. In darkness, rates of respiration were approximately 1 μmol m^{-2} s^{-1} and were not significantly different between hybrids; neither was the initial slope of the light response curves, *i.e.* the apparent quantum yields, although that of SH was greater than Sungro. At irradiances >700 μmol m^{-2} s^{-1}, Pn of SH exceeded that of Sungro; photosynthesis of SH was not saturated at 1500 μmol m^{-2} s^{-1}, whereas that of Sungro was. Thus, under well-watered conditions, SH had a substantially greater capacity for CO_2 assimilation than Sungro.

Figure 2, a and b, shows the rate of CO_2 assimilation as a function of ψ for two experiments. As shown previously, SH had a greater rate of Pn than Sungro in well-watered conditions and under water stress in one of the experiments (Fig. 2b). In both hybrids, the P_{max} decreased linearly with decreasing ψ. The substantially greater P_{max} over the range of ψ in the first, compared to the second experiment, may be related to the much brighter conditions during growth of the plants in the first experiment. Normalized to P_{max}, assimilation decreased curvilinearly with ψ (Fig. 2c). The substantially larger P_{max} of well-watered plants of SH compared with Sungro decreased as ψ decreased; below −1.7 MPa the response was similar for both hybrids (Fig. 2, a and c).

Differences in assimilation between hybrids were not related to their g_s, determined for the whole leaf sample, which were not statistically different over the range of ψ. g_s of both hybrids was very small (0.04 mol m^{-2} s^{-1}) below −1.2 MPa (Fig. 3a). The greater photosynthetic capacity of SH than Sungro at all but the smallest g_s is also emphasized in Figure 3b. Autoradiography of leaf pieces treated in the same way as those used for Pn measurements, but exposed to $^{14}CO_2$ (Fig. 4a), show that there is only a small variation in the distribution of radioactivity across the surface, with resolution of between 1 and 0.1 mm which allows minor veins, glands, and hair bases to be distinguished. The analysis of film density by chromatographic scanner (Fig. 4b) supported the visual impression. From correlation of the ^{14}C in the tissue, with the mean g_s and with the film density, an estimate of the variation in photosynthesis and conductance over areas of 0.01 mm^2 and greater was made. The range of Pn in control leaves was about 1 μmol m^{-2} s^{-1} at an average rate of 20 μmol m^{-2} s^{-1}, and g_s over the mesophyll between veins (g_s about 0.5 mol m^{-2} s^{-1}) was 0.05 mol m^{-2} s^{-1} (*i.e.* a 10% variation). In leaves stressed to −1 MPa, Pn was 12 μmol m^{-2} s^{-1} with a variation of 2 μmol m^{-2} s^{-1}, and g_s was approximately 0.07 mol m^{-2} s^{-1} with a variation of 0.02 mol m^{-2} s^{-1} (approximately 30%). In more severely stressed leaves, variation in Pn was <1 μmol m^{-2} s^{-1} in 5 μmol m^{-2} s^{-1}, and variation in g_s (0.01 mol m^{-2} s^{-1}) was estimated to be about 50%. Irregularities of contact between leaf and film and differences in the thickness of material absorbing ^{14}C β-emissions may also contribute to the variation.

Visual examination of the penetration of solutions into leaves suggested that in well-watered leaves the stomatal pores were open uniformly over the leaf surface. With stress, more penetrating solvents were required to enter the leaf, but generally solutions entered uniformly, as expected if localized

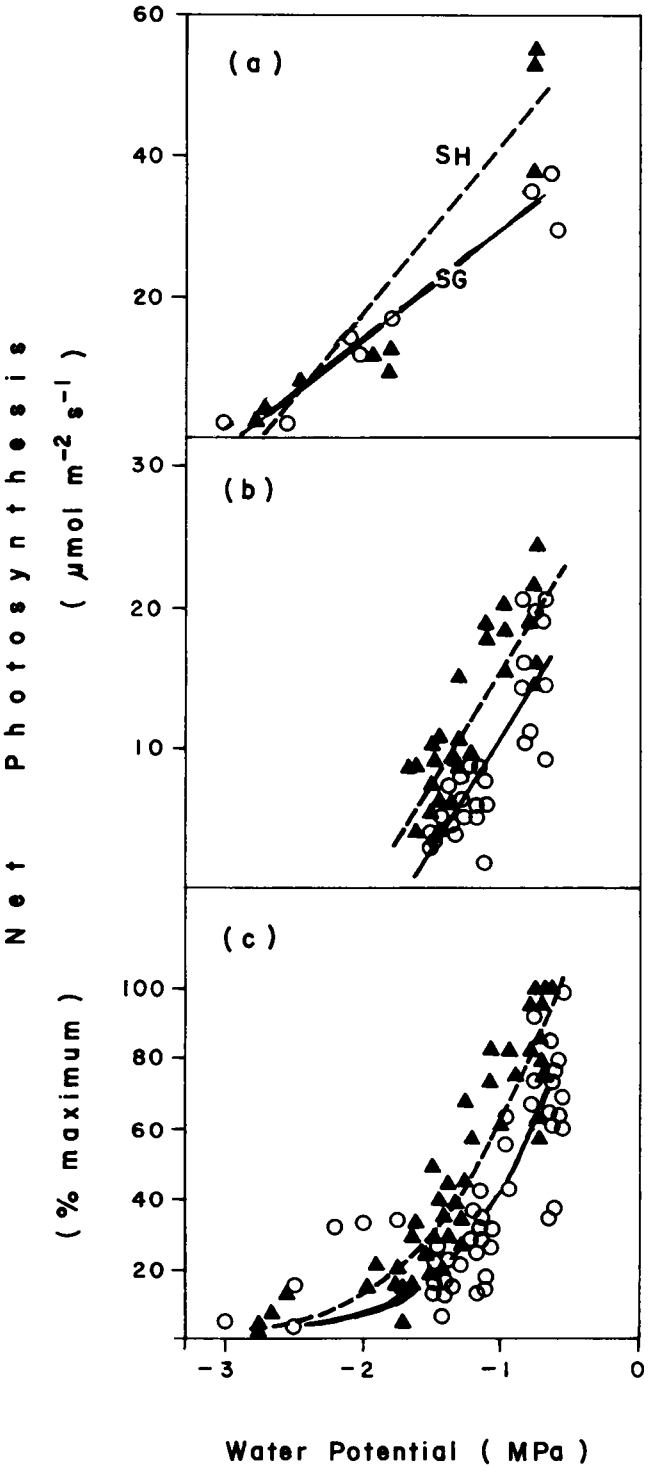

Figure 2. The response of Pn of sunflower hybrids SH (▲) and Sungro (○) to decreasing leaf ψ in two different experiments. Plants were grown in bright, natural daylight (a) or a controlled environment (b). The relation of photosynthesis, expressed as a proportion of the maximum rate, to ψ is shown in c.

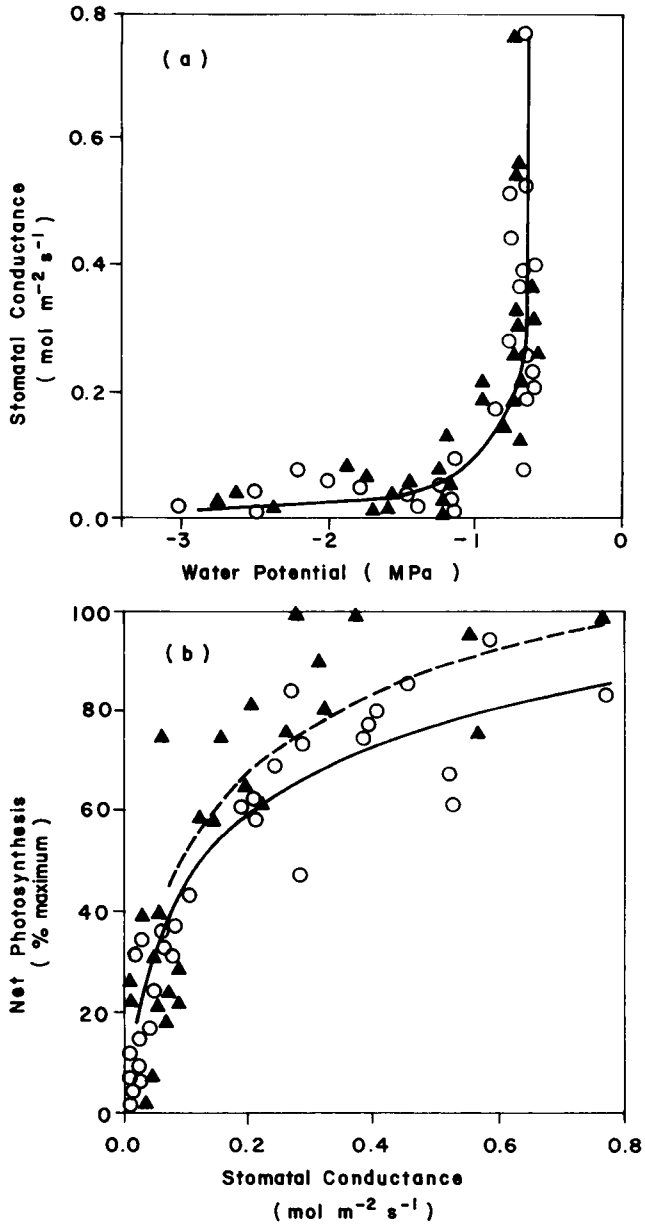

Figure 3. a, g_s as a function of leaf ψ; b, Pn, expressed as a percentage of the maximum rate, in relation to g_s. Symbols as in Figure 2. SH % Pn = 103.9 + 22.06 ln g_s. Sungro % P_s = 90.86 + 19.5 ln g_s.

stomatal closure did not occur. This, together with the analysis of variation in rates of Pn and g_s by $^{14}CO_2$ feeding, strongly suggests that irregular spatial variation in stomatal opening is not responsible for the observed differences in the relation between Pn and Ci.

The carboxylation efficiency was greater in young leaves of SH than Sungro; it decreased substantially (Fig. 5) with decreasing ψ similarly in leaves of both hybrids. However, in leaves >18 d after full expansion, there were differences in carboxylation efficiency related to the onset of senescence, although the effect of stress was still apparent (data not shown).

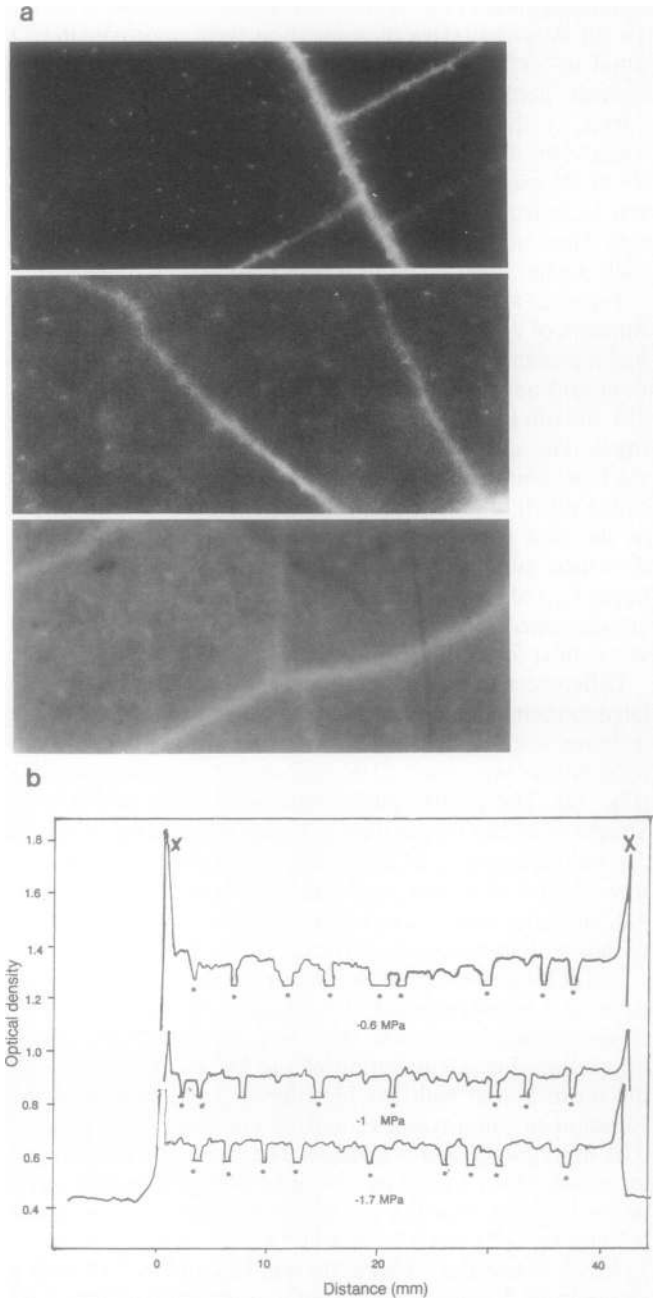

Figure 4. a, Autoradiographs of leaves of sunflower SH exposed to $^{14}CO_2$ for 30 s under standard conditions, before freezeclamping, followed by exposure to X-ray film while frozen. a, Control leaf, ψ = −0.6 MPa; b, leaf stressed to ψ = −1.2 MPa; c, leaf stressed to ψ = −1.8 MPa. Bar = 1 mm. b, Densitometer scans across the width (4.4 cm) of autoradiographs made from leaf samples freeze clamped in the gas exchange chambers. The position of the veins is shown by *. Increased exposure to radioactivity at the cut edges of the leaves is seen as greater density at X.

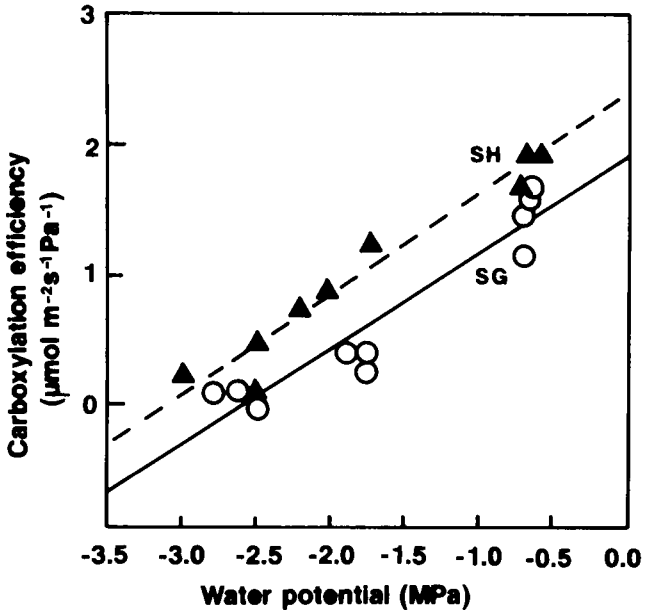

Figure 5. Carboxylation efficiency of SH (▲) and Sungro (SG, ○) in relation to the ψ of young, fully expanded leaves. Sungro: carboxylation efficiency = 0.077 ψ + 0.257; r^2 = 0.952. SH: carboxylation efficiency = 0.072 ψ + 0.187; r^2 = 0.850.

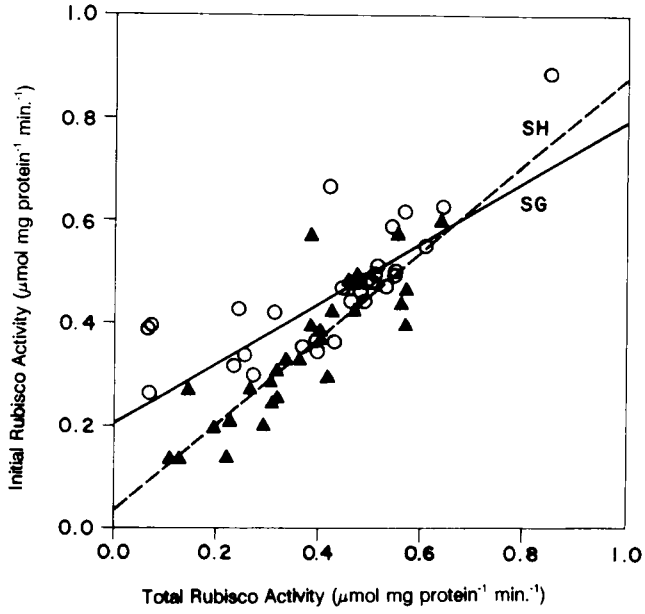

Figure 6. The initial activity of Rubisco immediately following extraction from leaves of SH (▲) and Sungro (SG, ○) related to total measured activity.

Chl, Soluble Protein, and Rubisco Amount and Activity

Leaves of well-watered Sungro did not contain significantly more Chl and tsp per unit area of leaf than SH, but their ratio of Chl *a* to Chl *b* was smaller (Table I). The ratio of Chl to tsp did not differ significantly between the two hybrids (in experiment 1, Sungro: Chl = 0.299 + 0.0326 tsp, r = 0.715; SH: Chl = 0.340 + 0.025 tsp, r = 0.681). Water stress did not significantly affect the Chl *a*/*b* ratio nor the Chl or tsp content.

The amount of Rubisco protein per unit of leaf area did not differ significantly between the two genotypes. The correlation of Rubisco protein with tsp (Sungro: Rubisco = 0.43 tsp − 0.94, r = 0.788; SH: Rubisco = 0.42 tsp + 5.78, r = 0.682) showed that 42% of the tsp was Rubisco, with no significant difference between the hybrids. Water stress did not affect the proportion. The initial activity of Rubisco was not significantly different, nor was there an effect of ψ. The activity of Rubisco extracted from both varieties did not increase with incubation with CO_2 and Mg^{2+} in the assay medium. The fully activated specific activity of Rubisco was 0.017 ± 0.005 and 0.014 ± 0.004 μmol CO_2 (mg Rubisco protein)$^{-1}$ s^{-1} in Sungro and SH, respectively. There was no difference in the initial activation state of the enzyme in the two varieties; it averaged 108% in Sungro and 97% in SH (Fig. 6); nor did ψ have an affect on initial or total activity in either variety. Preliminary tests showed that activation for up to 10 min did not increase the measured activity significantly, but thereafter activity decreased slowly, despite the presence of a protease inhibitor.

Table I. *Characteristics of the Young, Fully Expanded Leaves of Two Sunflower Hybrids Differing in Photosynthetic Rate, Grown under Well-Watered Conditions*

Mean of five replicates ± SE.

Parameter	Sungro	SH
Ψ (MPa)	−0.68 ± 0.04	−0.70 ± 0.02
Chl		
g·m^{-2}	0.51 ± 0.05	0.48 ± 0.02
mg·g^{-1} dry wt	14.3 ± 1.3	15.2 ± 0.3
Chl *a*/*b* ratio	2.76 ± 0.09	2.81 ± 0.05
tsp		
g·m^{-2}	6.4 ± 0.89	6.0 ± 0.3
mg·g^{-1} dry wt	178.8 ± 23.5	189.2 ± 1.7
Cell No.		
10^{10}·m^{-2}	3.81 ± 0.19	4.71 ± 0.14
10^6·g^{-1} dry wt	1081 ± 38	1436 ± 28

The amount of RuBP extracted from the tissues under comparable Ci (25 to 28 Pa, data not shown) and quantum flux was significantly greater in unstressed leaves of SH compared with Sungro (Fig. 7). It decreased by 65% in SH and 44% in Sungro as ψ decreased from −0.6 to −1.6 MPa so that there was no difference in the amount of RuBP in stressed leaves. The decrease in Pn with decreasing RuBP was sigmoidal, with SH having more RuBP than Sungro at the highest rates of assimilation (Fig. 8).

Cell Numbers and Sizes

The number of cells per unit area and per unit dry mass of leaf was significantly greater in SH than in Sungro (Table I). Fully turgid cells of Sungro were substantially larger than those of SH (Fig. 9). Cell volume decreased with decreasing ψ slightly more in Sungro than in SH; comparing −0.6 with −2.0 MPa, Sungro cells decreased in volume by 45 compared with 37% in SH. The osmotic potential decreased from

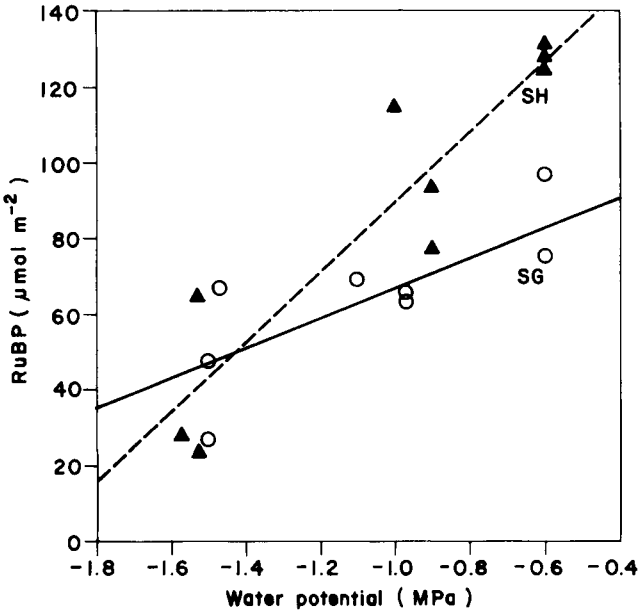

Figure 7. The amount of RuBP in leaves of SH (▲) and Sungro (SG, ○) in relation to their ψ.

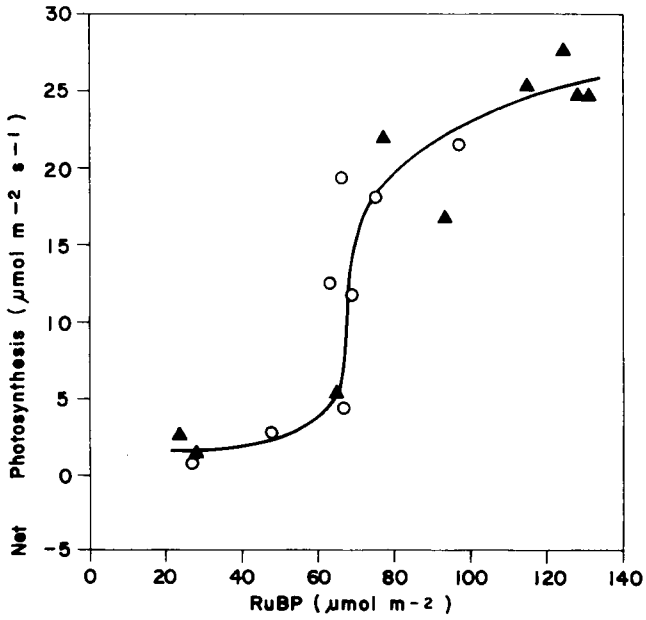

Figure 8. The relation between Pn and the RuBP content of leaves of SH (▲) and Sungro (○).

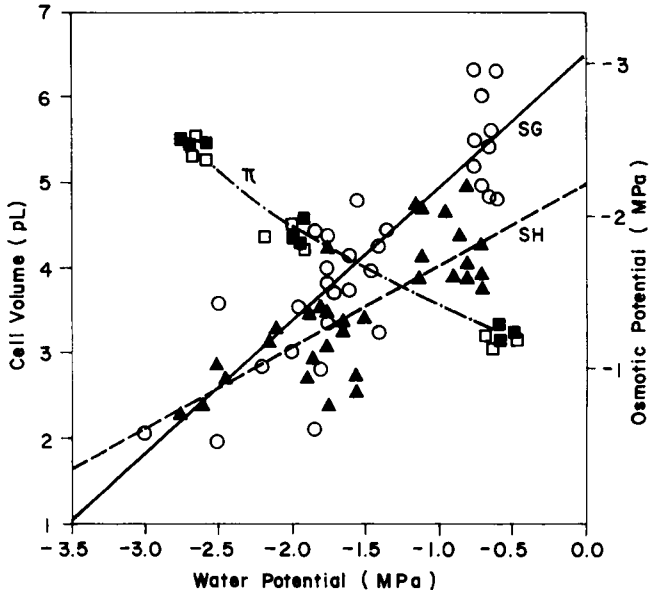

Figure 9. The volume of leaf cells from SH (▲) and Sungro (SG, ○) in relation to the leaf ψ. The osmotic potentials (Δ) of SH (■) and Sungro (□) are shown.

−1.3 MPa in turgid cells of both varieties to −2.2 MPa at −2.0 MPa ψ.

DISCUSSION

Under the experimental conditions and without water stress, fully expanded leaves of SH had greater rates of Pn than Sungro at saturating CO_2 and large photon flux (Fig. 1). Also, the carboxylation efficiency of SH was greater than that of Sungro. Therefore, factors related to the ability of the tissue to absorb CO_2 and also in the capacity of the CO_2 assimilation system must differ between the hybrids (28, 29). The cause of the difference in Pn is to be sought in the tissue composition and structure which also determine the response of the plant to water deficits. There was no indication that the gross amounts of Chl, the Chl *a*/*b* ratio, or the amount of tsp could account for the greater rate of CO_2-saturated Pn in SH compared with Sungro. Also, the ratio of Rubisco protein to tsp was constant, within the errors, and the amount of total extracted activity of Rubisco was the same in the two hybrids. Differences between the hybrids could be caused by different activation states, but comparison of Rubisco activity immediately after extraction and following activation showed that Rubisco was not differentially activated in the two genotypes. Calculation of the specific activity of the extracted Rubisco gave Sungro an activity of 0.017 ± 0.005 and SH 0.014 ± 0.004 μmol CO_2 mg Rubisco protein^{-1} s^{-1} (*cf.* ref. 18), suggesting that the activity was not responsible for differences in CO_2 assimilation. Thus, the differences must lie in other parts of the photosynthetic carbon reduction cycle, *e.g.* in its links to the energy-transducing system of the thylakoids or in export of products from the chloroplasts (5, 16). The differences may occur in the amounts or activities of carbon cycle enzymes other than Rubisco, involving feedback regulation via prod-

ucts of the reactions, energy status of the thylakoids, etc. (26, 28, 29), which may be reflected in the pool sizes of RuBP in the hybrids.

The measurements showing that SH had considerably more RuBP per m^2 of leaf than Sungro at Ci approaching saturation (approximately 28 Pa) are important, because large RuBP pool size may be expected to support a faster rate of CO_2 assimilation. This observation implies that faster rates of synthesis of RuBP or the ability to maintain large stromal concentrations of RuBP are major factors determining the difference in ability of related plants to support greater CO_2 assimilation (5, 26, 28, 29). A decrease in RuBP from the maximum observed in SH to that found in Sungro (32%) is related to a 20% loss in assimilation capacity. At large Pn, SH has more RuBP (per unit of Pn and of Rubisco) than Sungro (Fig. 8). This would allow the enzyme in SH to operate with a larger ratio of substrate than in Sungro.

The concentration of Rubisco enzyme sites in the chloroplast stroma was calculated, assuming that Rubisco has a molecular mass of 550,000 g mol^{-1} and eight substrate-binding sites per mol of enzyme protein, and that the volume of the chloroplast stroma is 1.2×10^{-5} m^3 m^{-2} leaf area (11). The maximal concentration of Rubisco catalytic sites is approximately 4 mol m^{-3} of stroma. The equivalent RuBP concentration calculated from our experiments is approximately 8 mol m^{-3} in well-watered plants at large Pn (*i.e.* 2 mol RuBP per mol enzyme sites). The concentrations of RuBP in the chloroplasts of the two hybrids cannot be calculated accurately because their chloroplast volumes are not known. Estimates of the RuBP concentration in the chloroplast stroma of several species range from 0.6 to 4 and even as high as 12 mol m^{-3} (11, 28, 29). With a K_m for RuBP of 25 to 40 mmol m^{-3} (23, 26, 28) the enzyme should always be saturated with RuBP, but this is probably not the case, particularly with water stress when the concentration of RuBP decreases by 50% and Pn almost ceases. The required RuBP concentration for effective assimilation *in vivo* is therefore approximately 50 to 100 times greater than *in vitro*; presumably efficient carboxylation requires a large concentration of RuBP in the stroma to ensure saturation of the enzyme catalytic sites. A large concentration may also be required to ensure adequate rates of diffusion of RuBP through the stroma.

The other major difference observed in the two hybrids is the number and sizes of cells per unit area of leaf; cells of SH were 20% smaller than those of Sungro when fully turgid. Although the transport of CO_2 to the chloroplast may limit Pn in deficient CO_2, it cannot be the limitation in saturating CO_2. However, smaller cell volume and shorter pathways for diffusion may allow faster transport (18, 22) of materials between cells or cell compartments in SH than in Sungro, *e.g.* of phosphate needed for ATP synthesis and therefore for RuBP regeneration and thus greater Pn.

With water stress, which decreases cell volume and ψ, the g_s of both SH and Sungro decreased similarly to the same minimal value (0.05 mol m^{-2} s^{-1}) at about −1.2 mPa ψ with a decrease of cell volume of approximately 11% in SH and 13% in Sungro. However, the decrease in Pn at low ψ was not caused by low g_s because maintaining Ci at saturation (data not given) did not increase Pn (5, 16), suggesting that it is not inadequate Ci that limits Pn but metabolic damage.

The calculation of Ci assumes that the g_s is uniform across the leaf. If stomata on small areas are closed (patchiness), Pn decreases, but the calculated Ci is incorrect (see ref. 7). Decreasing Pn at a given Ci is characteristic of the effects of ψ on the Pn/Ci relationship (Fig. 1a) and in other studies (3, 15, 16, 25). Visual and semiquantitative examination of the uptake of $^{14}CO_2$ by leaves of the sunflower varieties with different ψ showed that the distribution of radioactivity within the mesophyll was rather uniform (Fig. 4), even at resolution as small as 0.1 mm, clearly not as great as expected if some areas of the leaf had large g_s and others had closed stomata, in contrast to the effects of mild water stress on bean (25). Thus, patchy stomatal closure is not considered responsible for the reduction of Pn or of the altered relation to Ci.

In water-stressed leaves of both hybrids of sunflower, the primary site of limitation of Pn seems related to RuBP content, not to the amount of Chl, tsp, or Rubisco protein, because these changed little over the 4 to 6 d of stress, nor in the activation state of Rubisco or its specific activity, similar to the response of Rubisco in mildly stressed bean leaves (25; see also refs. 27, 28). A minimum concentration of RuBP of approximately 50 μmol m^{-2} corresponds to a ψ of about −1.6 MPa and to a 27% decrease in cell volume for both genotypes, similar to the changes that affect Pn substantially in isolated cells (14). The RuBP concentration was approximately 4 mol m^{-3}, *i.e.* 1 mol RuBP per mol sites at very small Pn. Evidently, RuBP content is closely related to the rate of Pn, and RuBP regeneration is the most likely site of inhibition by decreased ψ or osmotic potential; under water stress, RuBP synthesis probably depends on the rate of supply of ATP to the photosynthetic carbon reduction cycle (3, 4, 5, 16). Chloroplast-coupling factor was inhibited (5) in this range, possibly because of increased ion (particularly Mg^{2+}) concentration in the chloroplast stroma.

We conclude that the greater Pn of SH than Sungro in high CO_2 and in bright light is related to smaller size of leaf cells and, particularly, to a larger amount of RuBP per unit area of leaf. Water stress decreased the assimilation of the hybrids similarly, by decreasing their RuBP content rather than via stomatal or Rubisco regulation.

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