and sunflower (Figure 2). This illustrates that assimilation rates are controlled by the mesophyll factors also and the proposed method can be conveniently used for rapid evaluation of variations in the mesophyll efficiency of a given leaf.

If higher carboxylation efficiency determines a better mesophyll factor, a leaf that shows higher dA/dC_i should have lower C_i/G_s ratio. A strong inverse relationship ($R^2 = 0.87$) clearly suggests that C_i/G_s does indeed reflect the mesophyll efficiency (Figure 4).

Since the proposed method only involves the measurements of gas exchange traits, estimation of mesophyll efficiency can be done relatively rapidly, especially while evaluating the germplasm lines. Further evaluation of the approach in relation to the carboxylation efficiency associated with RuBisCO has been carried out (Krishna Prasad *et al.* 1996, this issue).

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Regulation of carboxylation by RuBisCO content and its efficiency in sunflower and soybean

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In addition to the CO_2 diffusive processes, the mesophyll efficiency also determines the variability in carbon assimilation rates (A) of leaves. Mesophyll efficiency as calculated by the ratio of the intercellular CO_2 concentration to the stomatal conductance (C_i/G_s) offers proof for the mesophyll control of photosynthesis. The mesophyll factors are, in turn, largely associated with the Ribulose 1,5-biphosphate carboxylase (RuBisCO) content and/or its efficiency of carboxylation. To evaluate the role of RuBisCO in determining the variability in mesophyll efficiency, we studied these parameters in a few genotypes of sunflower and soybean, showing significant genetic variability in gas exchange parameters and in RuBisCO content. The efficiency of RuBisCO was computed by the ratio of A per unit RuBisCO. A among soybean genotypes showed a positive relationship with RuBisCO content, while in sunflower genotypes it was related to the efficiency of RuBisCO. Its content in soybean genotypes and the efficiency among sunflower genotypes showed a strong relationship with the C_i/G_s ratio, reiterating the role of RuBisCO in regulating the mesophyll efficiency. We conclude that the mesophyll efficiency is regulated by RuBisCO content and/or its efficiency across the genotypes of sunflower and soybean.

A considerable genotypic variability in photosynthetic CO_2 assimilation rate per unit leaf area (A) has been reported in many crop species[1–5]. But, exploiting this genetic variability for enhancing A, by conventional breeding approaches has met with little success[6,7]. This is partly due to the complex genetic control and the lack of adequate knowledge of limiting factors of photosynthesis under a given environment.

Under optimum growing conditions, the realized assimilation rates are often a few degrees of magnitude less than the potential of chloroplasts[8,9]. The primary limitation to achieve these potential assimilation rates is the lack of substrate CO_2 availability at the carboxylation site due to stomatal diffusive resistances for CO_2 (refs 10–14).

Apart from the stomatal factors, mesophyll also plays an important role in influencing the variability in A. A linear relationship between the carboxylation efficiency as computed by the initial slope of the CO_2 response curve (dA/dC_i) and assimilation rate suggests that A is controlled by the mesophyll (RuBisCO) factors[9,15,16]. In an earlier paper, we reported that the ratio of the intercellular CO_2 concentration (C_i) to stomatal conductance (G_s) is a good reflection of the mesophyll efficiency for carboxylation[17]. A strong inverse relationship between A and C_i/G_s ratio suggests that A is controlled by the mesophyll efficiency.

At a given G_s, the fixation of CO_2 depends on the carboxylation associated with RuBP carboxylase/oxygenase (RuBisCO), the photochemical reactions of the lamellar system, and the regeneration of inorganic orthophosphate (P_i) associated with end product synthesis. Recent studies has shown that the assimilation rates at ambient CO_2 concentrations are not limited by photochemical reaction[18–20] or by P_i recycling[21,22].

Adopting various approaches, it has been demonstrated that assimilation rates could be under the control of RuBisCO (ref. 23). The role of RuBisCO in regulating the variations in assimilation rates has been proved using transgenic plants with antisense constructs for the small subunit of RuBisCO (refs 24–26). Further, the variation in *A* of the leaves at different canopy positions has been shown to be dependent on RuBisCO content[27–29]. These data signify the importance of RuBisCO content. However, we report from the present investigation that variation in carboxylation efficiency could also be brought about by the efficiency of RuBisCO in some species such as sunflower. On the other hand, in species that intrinsically have lower RuBisCO content such as soybean, genetic variability in RuBisCO content may bring in variability in carboxylation efficiency and hence in *A*.

A few genotypes of sunflower (*Helianthus annuus* L.) and soybean (*Glycene max*) (refer Tables for the names of genotypes) were collected from the germplasm collections of the University of Agricultural Sciences, GKVK, Bangalore. Seeds were sown in carbonized rubber containers (15 kg capacity) and seedlings were subsequently thinned to maintain only two plants per pot. Plants were watered twice daily to maintain the pots at field capacity. After 35 days of growth, gas exchange, total soluble protein and RuBisCO contents were quantified using 0.5 g of the top fully expanded leaves.

Carbon assimilation rates (*A*), stomatal conductance (G_s) and intercellular CO_2 concentration (C_i) were measured on the top fully expanded leaves of the genotypes using a portable photosynthesis system (ADC model LCA-2, UK). All observations were recorded between 9:30 and 11:00 AM on a bright sunny day with a photon flux density of around 1500 μ moles m^{-2} s^{-1} quanta. The vapour pressure deficit (VPD) was maintained between 12 and 15 mbars by suitably adjusting the flow of dry air through the Air Supply Unit. Temperature of the cuvette was maintained at 27 ± 1°C. Immediately after recording the gas exchange parameters, the leaves were detached and used to quantitate the total soluble proteins and RuBisCO content.

A simple dye binding technique was adopted to quantitate the total soluble proteins in leaf extracts[29]. Area of a known weight of deveined leaf blade was recorded before homogenizing in 100 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA, 2% PVP and 1 mM PMSF. The homogenate was centrifuged at 10,000 *g* for 10 min. An aliquot of the clear supernatant was used to estimate the proteins. The absorbance values were compared with standard curve developed using bovine serum albumin (Sigma).

RuBisCO holo enzyme was purified from rice leaves and injected into New Zealand rabbits to develop polyclonal antibodies. Using these antibodies, we standardized an indirect ELISA procedure to quantify the enzyme content in the plant crude extract. A crude extract of total soluble protein was prepared as explained above and used for the immuno assay.

A significant genetic variation in *A*, G_s and C_i exists among the genotypes of sunflower and soybean (Tables 1 and 2). Stomatal conductance showed a significant positive correlation with *A* among the genotypes of both the crops ($r = 0.8$).

If stomata were the only controlling factor, there must have been no change in C_i levels, as increase in *A* would be proportional to the increase in G_s. A significant genotypic variation in C_i among the genotypes of both the crops and a strong inverse relation between *A* and C_i/G_s indicates that besides stomatal factors, the mesophyll efficiency also contributes significantly to the

Table 1. Regulation of carboxylation in sunflower*

Genotype	*A*	G_s	Protein (P) (g m^{-2} LA)	RuBisCO (R) (g m^{-2} LA)	A/R	R/P (%)
Acc 1610	27.2	1097	2.9	0.91	29.9	31.4
Acc 1599	27.2	848	2.1	0.72	37.7	34.3
Acc 1600	23.0	849	2.5	1.09	29.1	43.6
Acc 1648	22.6	963	2.1	0.78	29.0	37.1
Acc 1616	22.2	877	3.5	0.79	28.1	22.6
M 787-7-2	20.8	863	2.4	0.84	24.7	35.0
Acc 1630	20.2	621	2.4	0.99	20.3	41.3
62-B	19.6	814	2.3	0.79	24.9	34.3
339-B	16.3	438	2.3	1.03	15.9	44.8
Mean	22.1	819	2.5	0.88	26.6	35.2
CD ($P = 0.05$)	1.2	5	0.13	0.15	–	–

*Assimilation rate (*A*), stomatal conductance (G_s), soluble protein, RuBisCO content and its efficiency in a few genotypes of sunflower.
Gas exchange parameters were recorded on the top fully expanded leaf on a bright sunny day (1500 μ mole m^{-2} s^{-1}). Leaf protein and RuBisCO content were quantified immediately in the same leaf ($n = 10$).
A = μ moles m^{-2} s^{-1}; G_s = mmoles m^{-2} s^{-1}.

Table 2. Regulation of carboxylation in soybean*

Genotype	A	G_s	Protein (P) (g m^{-2} LA)	RuBisCO (R) (g m^{-2} LA)	A/R	R/P (%)
IS-87-60	23.4	1025	3.20	0.89	26.3	27.8
Punjab-1	22.7	983	2.94	0.80	28.3	27.2
MACS-409	22.0	864	3.00	0.88	25.0	29.3
Seln. 5	21.4	1016	3.10	0.87	24.5	28.1
Pusa-16	20.8	1035	3.15	0.82	25.4	26.0
JS-75-46	19.5	841	2.65	0.72	27.0	27.2
PK-1029	19.4	660	1.80	0.50	38.7	27.7
Pusa-20	18.8	714	2.59	0.66	28.4	25.5
JS-87-59	18.7	780	2.74	0.58	32.2	21.2
KB-92	17.0	671	1.74	0.48	35.4	27.6
DS-83-12-2	16.9	576	1.95	0.44	38.5	22.6
Mean	20.1	833	2.62	0.69	29.97	26.4
CD ($P = 0.05$)	0.8	6	0.044	0.02	–	–

*Assimilation rate (A), stomatal conductance (G_s), soluble protein, RuBisCO content and its efficiency in a few genotypes of soybean.
Gas exchange parameters were recorded on the top fully expanded leaf on a bright sunny day (1500 μ mole m^{-2} s^{-1}). Leaf protein and RuBisCO content were quantified immediately in the same leaf ($n = 10$).

variations in A (ref. 17). We have reported that the ratio of C_i to G_s was closely associated with the initial slope of CO_2 response curve (dA/dC_i), further substantiating the importance of mesophyll efficiency in regulating A.

Since mesophyll efficiency is primarily associated with carboxylation efficiency, which in turn is predominantly controlled by RuBisCO, we quantified RuBisCO content in sunflower and soybean genotypes. A significant genotypic variation was seen in total soluble protein as well as RuBisCO content (Tables 1 and 2). Sunflower genotypes, on an average, had significantly higher RuBisCO content (0.88 g m^{-2} LA) than soybean genotypes (0.69 g m^{-2} LA). There was only a marginal difference in total soluble protein content between the crops. However, genotypic differences were evident especially in soybean. The allocation of total soluble protein to RuBisCO was markedly higher in sunflower (35.6%) than soybean (26.4%). Mean values of total soluble protein and RuBisCO of the species indicate that higher A in sunflower could mainly be due to higher RuBisCO content per unit leaf area.

Regression lines were plotted to examine the extent of control of A by RuBisCO content among the genotypes (Figure 1). The variation in RuBisCO content among the sunflower genotypes did not correspond to variations in A (Figure 1 *a*). On the other hand, a positive relationship between A and RuBisCO content ($R^2 = 0.82$) was recorded among soybean genotypes (Figure 1 *b*).

The ratio of A to RuBisCO content per unit leaf area (A/R) was computed to arrive at the *in vivo* efficiency of RuBisCO (Tables 1 and 2). Sunflower genotypes, on an average, showed lesser efficiency compared to soybean. Among sunflower genotypes, assimilation rates recorded a significant positive relationship with the efficiency of RuBisCO (Figure 2). In sunflower, the genotypic variation in A is predominantly controlled by the efficiency of RuBisCO (A/R) while among soybean genotypes, RuBisCO content seems to account for the variations in A.

The ratio of C_i/G_s, an estimate of mesophyll efficiency showed a significant inverse relationship with the efficiency of RuBisCO (A/R) in sunflower (Figure 3 *a*) while a similar relationship was evident with RuBisCO content among soybean genotypes (Figure 3 *b*). These plots illustrate that factors associated with RuBisCO regulate the variation in mesophyll efficiency and hence assimilation rates.

In recent years there has been an emphasis on understanding various components that limit photosynthesis. Although stomatal limitation has been shown to be the predominant limitation under non-stress conditions[11,12,14–16], variations in the mesophyll efficiency also have been implicated to play an important role in determining the variations in A. Relationships between A and dA/dC_i (refs 9,15) and/or C_i/G_s ratio[17] testify to the fact that assimilation rates are also controlled by mesophyll factors.

Out of a large number of independent and interdependent mesophyll factors, recent evidences suggest that neither photochemistry nor the P_i regeneration within the chloroplast are potential limitations for assimilation rates under normal conditions.

Significant increase in A when ambient CO_2 concentrations are increased is often quoted as a clear evidence to suggest that photochemical reactions do not limit A (refs 9,14,19). Stimulation of oxygen evolution at higher CO_2 concentrations indicates that the photochemical reactions have adequate capacity to support higher CO_2 fixation than ambient levels[18]. Increase in A and oxygen evolution with increase in temperature[22,31]

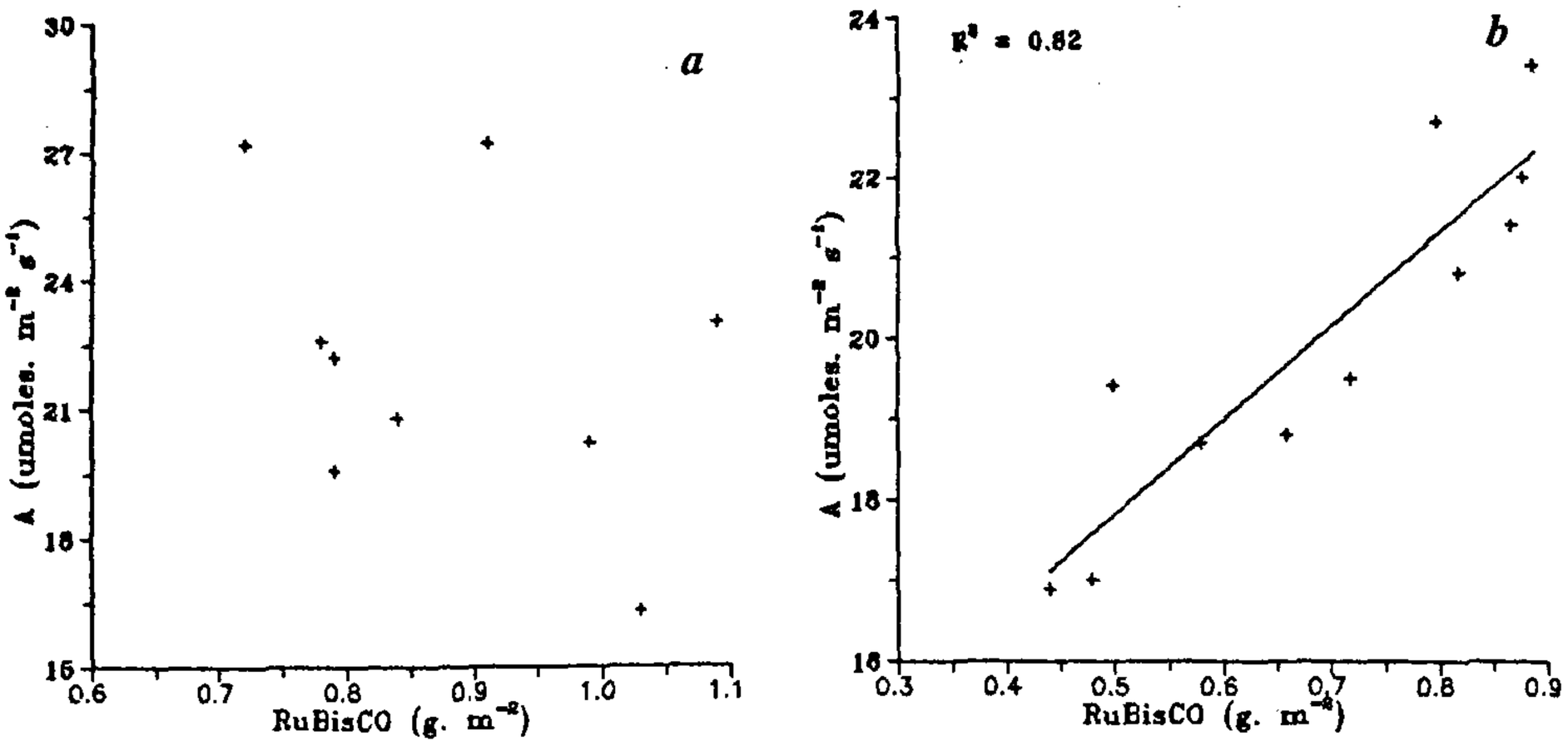

Figure 1. Relationship between assimilation rate (A) and RuBisCO content in (a), sunflower and (b), soybean genotypes. A was recorded on the top fully expanded leaves on a bright sunny day. The same leaf was used for the quantification of RuBisCO content by ELISA technique (each data point is a mean of three replications).

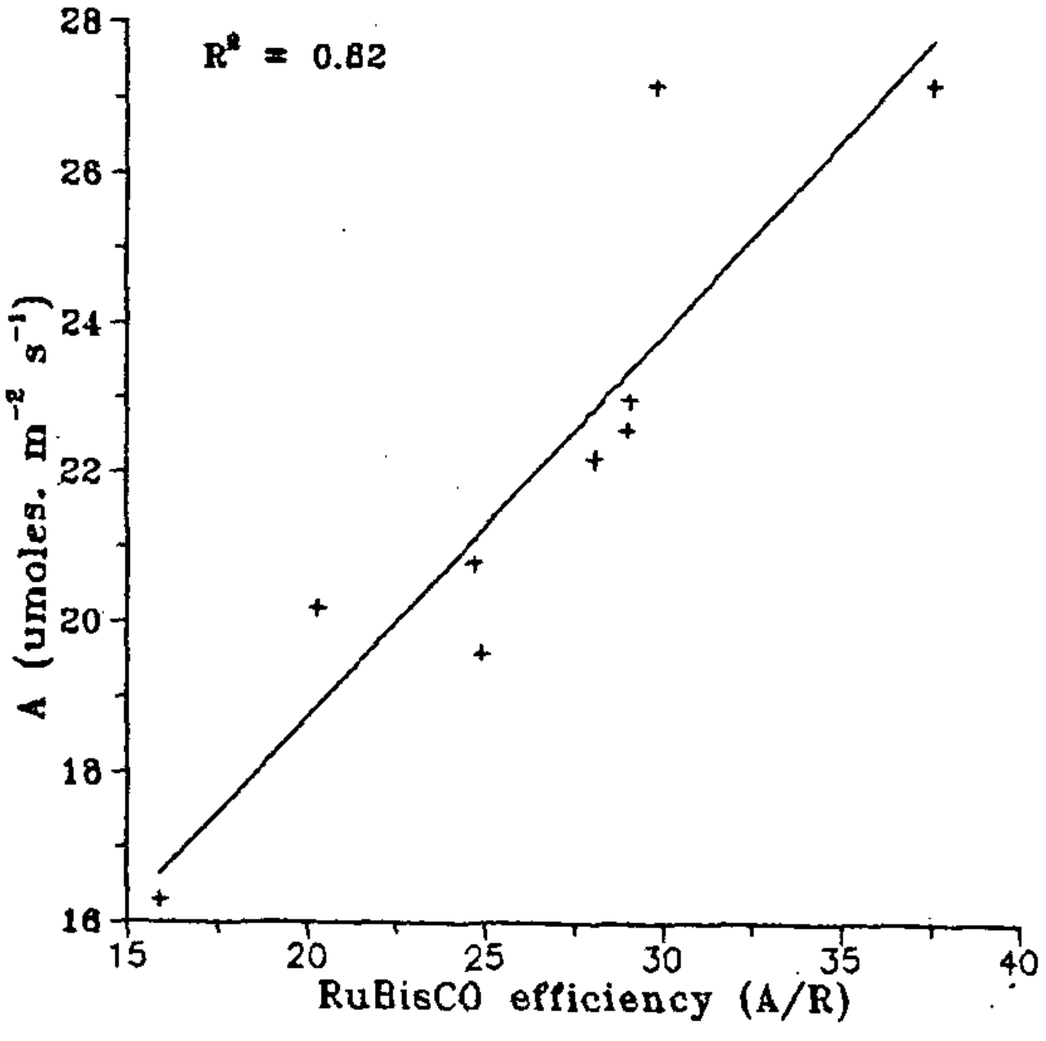

Figure 2. Assimilation rate (A) as a function of RuBisCO efficiency in sunflower. The RuBisCO efficiency was calculated as the ratio of A per unit RuBisCO expressed over unit leaf area.

and increased A with altered source to sink ratios reiterates that photochemistry does not limit A. However, photochemical reaction may be a limiting factor under low light intensities[32].

The regeneration of P_i during sucrose synthesis is an essential step in importing triose phosphate for end product synthesis. However, at ambient CO_2 levels, P_i regeneration is not considered to be an important limitation. Increase in A when CO_2 concentration is increased in short term experiments suggests that the P_i limitation may not be limiting A. Oxygen sensitivity of photosynthesis under ambient CO_2 concentrations also indicates that P_i regeneration does not limit A under ambient conditions[21,22,33].

Recent investigations using transgenic plants with antisense mRNA for the small subunit of RuBisCO suggest that RuBisCO content is an important determinant of carboxylation efficiency and hence of photosynthesis[24,34]. However, activation state and the kinetic constants of the enzyme have also been attributed to be limiting photosynthesis[35]. The transgenic plants with reduced levels of RuBisCO activase showed lesser activation state and hence lower photosynthetic rates[36].

In the present study, a significant genotypic variation in RuBisCO content was noticed. Variation in assimilation rate was linearly related to RuBisCO content only among soybean genotypes but not in sunflower (Figure 1). Besides RuBisCO content, its efficiency also may bring about variations in assimilation rate. The ratio of A per unit RuBisCO content is an indirect estimate of the efficiency of RuBisCO. The computed efficiency of RuBisCO showed a significant linear relationship with A among sunflower genotypes (Figure 2). These plots illustrate that carboxylation efficiency is controlled either by the RuBisCO content or its efficiency. If RuBisCO content is intrinsically low in a species, such as soybean, variations in A among its genotypes seems to be more under the control of RuBisCO content than its efficiency.

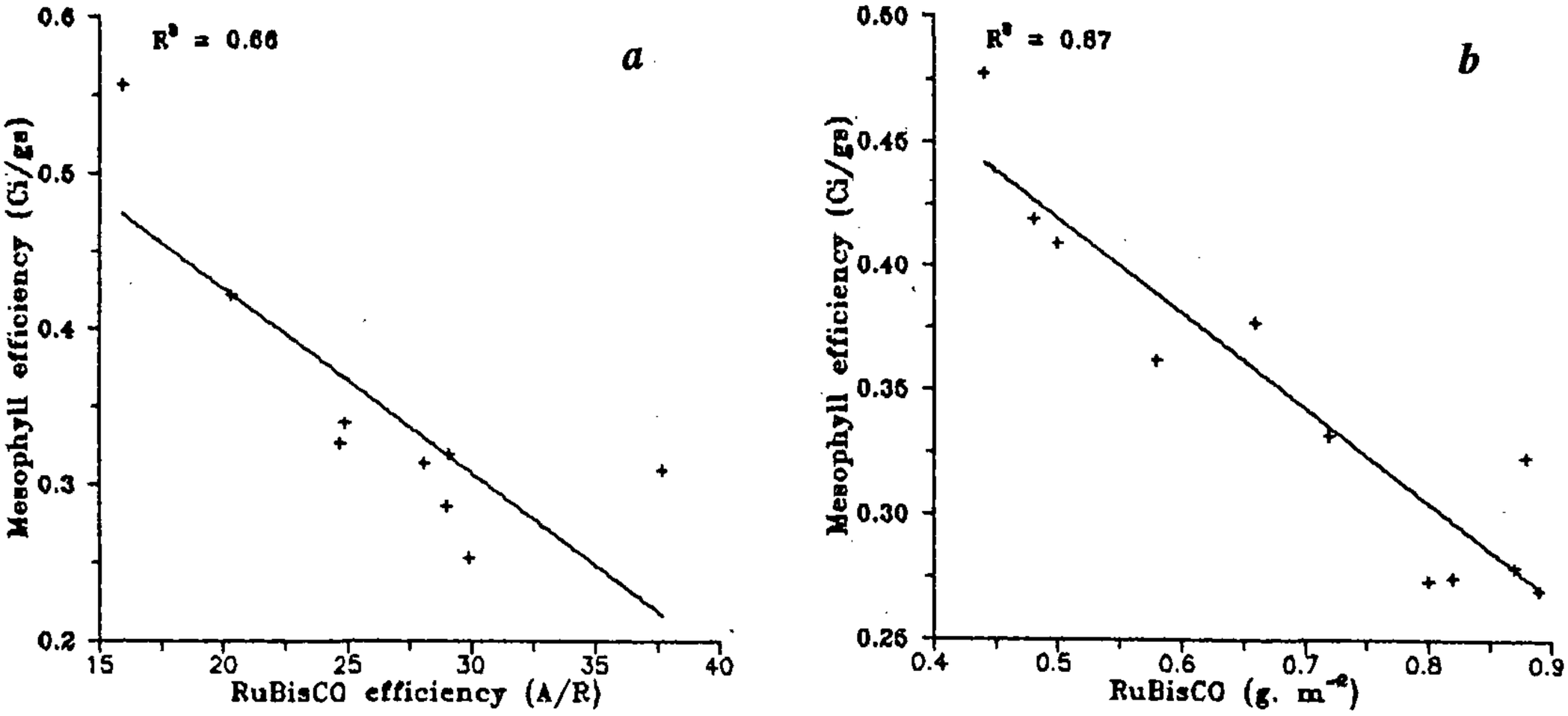

Figure 3. The mesophyll efficiency, as estimated by the ratio of C_i/G_s, is related to efficiency of RuBisCO in sunflower (*a*) and with the RuBisCO content in soybean (*b*).

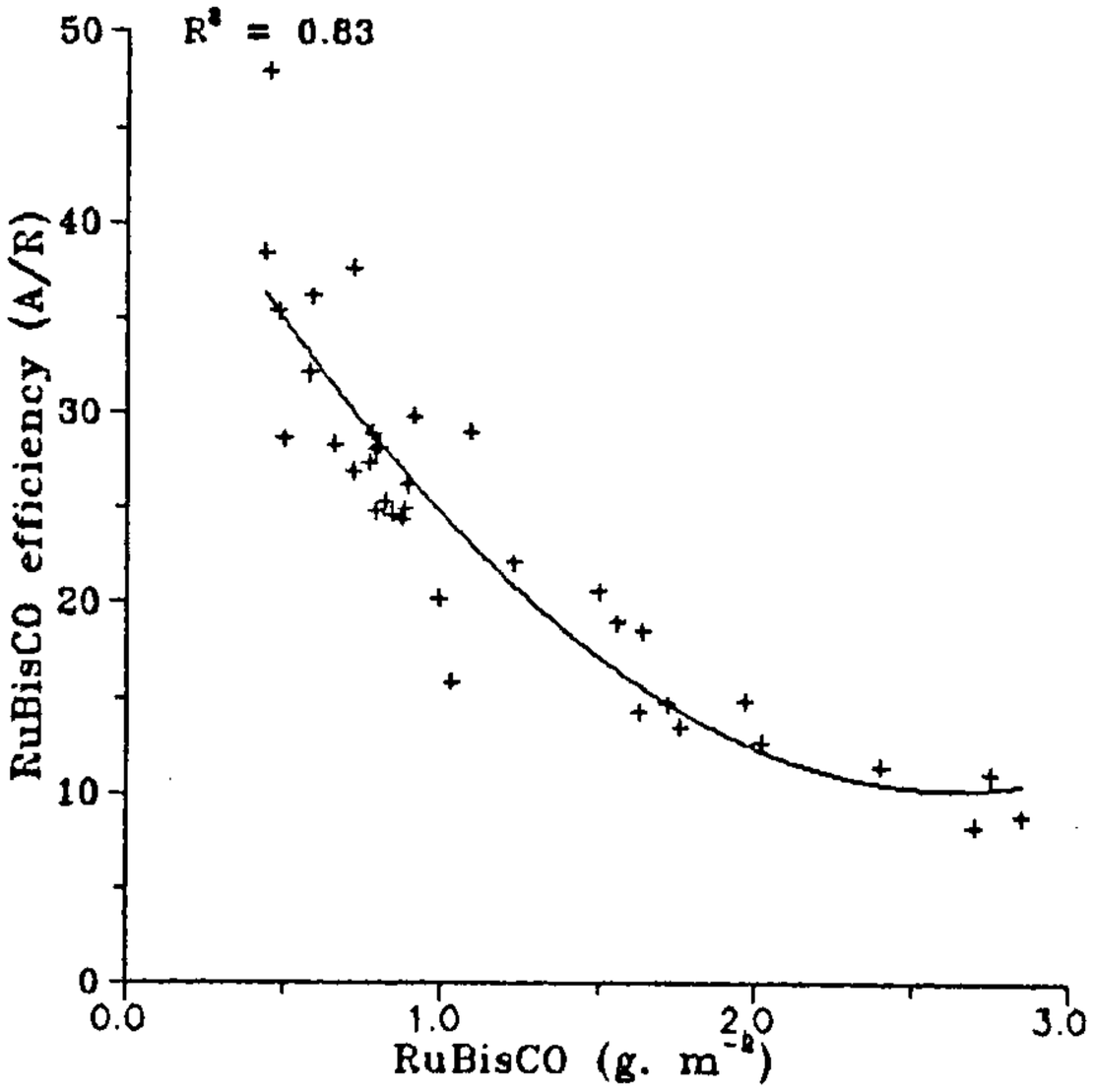

Figure 4. Relationship between the RuBisCO content and the efficiency of RuBisCO among the genotypes of soybean and sunflower.

The converse seems to be true in sunflower that has high RuBisCO content in which the control comes from the efficiency of RuBisCO rather than its content.

Further, the ratio of C_i/G_s, an estimate of mesophyll efficiency showed an inverse relationship with the efficiency of RuBisCO among sunflower genotypes, while a similar relationship of C_i/G_s ratio was noticed with the RuBisCO content among soybean genotypes (Figure 3). These data further suggest that the variations in mesophyll efficiency and hence *A* are related to either RuBisCO content or its efficiency.

While working with transgenic plants with altered RuBisCO levels, Hudson[37] showed that when RuBisCO content decreased, activation state of the enzyme increased. More recently, Mate and coworkers[36] showed an increase in RuBisCO content when activation state was decreased in transgenic plants with antisense genes for RuBisCO activase. In agreement with these findings, our data also showed an inverse relationship between RuBisCO content and its efficiency among soybean and sunflower genotypes (Figure 4).

Although RuBisCO content controls the variations in mesophyll efficiency in some species, selecting for more efficient RuBisCO seems to be a beneficial approach. This might also lead to the selection of higher nitrogen use efficient types. Nevertheless, it would be rewarding to evaluate reasons for why or how the RuBisCO content is down-regulated if the efficiency or activation state of the enzyme is increased.

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