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Variation in Carbon Isotope Discrimination and its Relationship to Specific Leaf Area and Ribulose-1,5-bisphosphate Carboxylase Content in Groundnut Genotypes*

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Abstract. Variation in carbon isotope discrimination (Δ) and ribulose-1,5-bisphosphate carboxylase–oxygenase (Rubisco) content per unit leaf area was examined in leaves from upper and lower positions in the canopy of six groundnut (*Arachis hypogaea* L.) genotypes, grown under irrigated and mild water-deficit conditions in the field. The leaf mass per unit leaf area (ρ_L) and soluble proteins in leaves were determined at 80, 96, 111 and 127 days after sowing (DAS), while Δ and Rubisco were determined at 80 DAS only. The mean Δ ranged from 18.2 to 20.2‰ among genotypes, representing a significant ($P < 0.01$) variation. Rubisco content per unit leaf area also varied significantly ($P < 0.01$) with genotype and leaf position. There was a trend to an increase in Rubisco content under water deficit, but the effects were not significant. Leaves at the top of the canopy had a higher Rubisco content and lower Δ, than leaves at the bottom of the canopy. Genotype × leaf position interaction was significant for Δ and Rubisco, indicating the importance of leaf position in selecting for water-use efficiency (W), using leaf traits in groundnut. Rubisco content and Δ were negatively related ($r^2 = 0.65$, $P < 0.01$). There was a significantly positive correlation between Rubisco content and ρ_L in the upper leaves ($r^2 = 0.60$, $P < 0.01$), but not in the lower leaves in the canopy. However, the overall relationship between Rubisco and ρ_L ($r^2 = 0.40$) was not as strong as it was between Rubisco and Δ. The results suggest that, in groundnut, the basis of genotypic variation in Δ was mostly (> 60%) attributable to Rubisco content per unit leaf area. In view of the leaf positional effects on Δ and Rubisco, the upper leaves in the canopy should be used for selecting genotypes for W based on leaf traits like ρ_L or Δ.

Introduction

In plant species with the C_3 photosynthetic pathway, variation in $^{13}C/^{12}C$ can be a useful indicator of water-use efficiency (Farquhar *et al.* 1982). A strong correlation between carbon isotope discrimination (Δ) and water-use efficiency (W, defined as g of dry matter produced per kg of water transpired by the crop) has been shown in crops of wheat (Farquhar and Richards 1984), barley (Hubick and Farquhar 1989), tomato (Martin and Thorstenson 1988) and groundnut (Hubick *et al.* 1986). In groundnut, genotypic variation for W and a significant correlation between W and Δ was shown in isolated plants grown in a greenhouse (Hubick *et al.* 1986), a canopy situation (Wright *et al.* 1988, 1994) and field-grown crops (Nageswara Rao *et al.* 1993).

A relationship between the ratio of internal to ambient CO_2 pressures (p_i/p_a) and Δ in C_3 plants (Farquhar *et al.* 1982) means that any factor affecting leaf conductance and assimilation rate in a disproportionate manner, thereby affecting p_i/p_a, will also affect Δ and W. In groundnut, genotypic differences in W appear to be associated with photosynthetic capacity per unit leaf area rather than stomatal factors (Hubick *et al.* 1986; Wright *et al.* 1988).

Low Δ and high W were shown to be associated with the leaf thickness (leaf mass per unit leaf area, or leaf superficial density, ρ_L) in groundnut, suggesting that an easily measurable parameter like ρ_L could be effectively used as a surrogate for Δ to assess W (Nageswara Rao and Wright 1994; Wright *et al.* 1994). Differences in photosynthetic rates were positively correlated with leaf thickness in alfalfa (Pearce *et al.* 1989), soybean (Dornhoff and Shibles 1976), oats (Criswell and Shibles 1971) and chickpea (Gupta *et al.* 1989), indicating that thicker leaves might have more photosynthetic machinery per unit leaf area. The CO_2 assimilation rate has been shown to be directly proportional to the amount of photosynthetic enzyme, i.e. ribulose-1,5-bisphosphate carboxylase–oxygenase, Rubisco (EC 4.1.1.39),

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present in leaves (von Caemmerer and Farquhar 1981). There is, however, very little information on the direct relationship between Δ and Rubisco levels in leaves.

Photosynthetic capacity declines with leaf age (Rawson *et al.* 1983; Evans 1986; Connor *et al.* 1993). Thus, leaf age (leaf position in the canopy) can potentially influence the selection of genotypes for *W* based on leaf traits, particularly if photosynthetic capacity is the major cause of variation in Δ or *W*. Information on the effect of leaf position in the canopy on Δ is essential if Δ and/or ρ_L traits are to be used in groundnut breeding programs.

The objectives of the present study were to: (*a*) examine the relationship between Δ and Rubisco content, and (*b*) study the effect of leaf position on Δ and Rubisco in a range of groundnut genotypes grown under irrigated and water-deficit conditions.

Materials and Methods

Crop Management

Six selected groundnut (*Arachis hypogaea* L.) genotypes were grown on an Alfisol field under adequately irrigated and water-deficit conditions during the post-rainy season (November–April) of 1991–1992 at ICRISAT Asia Center, central India.

The land was disc-ploughed after applying a basal dose of 48 kg ha^{-1} of phosphorus and prepared into broad beds of 1.2 m width, with 0.3 m furrows (15 cm deep) on either side of the bed. The seeds were treated with Captan* at a rate of 0.5 g kg^{-1} of seed and hand-sown at 10 cm spacing in four rows opened (parallel to furrows) at 0.3 m apart on each bed.

After sowing, the experimental area was sprayed with a pre-emergence herbicide (Alachlor at 4.0 L ha^{-1}) and irrigated with overhead sprinklers. After emergence, the crop was irrigated using a drip system, which delivered water at about 12 mm h^{-1}. The irrigation given to each treatment was measured with water meters (± 2% resolution). The crop received irrigation equivalent to 85% of the cumulative pan-evaporation, at weekly intervals until 70 days after sowing (DAS); subsequently, two irrigation regimes, i.e. adequate irrigation (T_1) and water-deficit treatment (T_2), were imposed until final harvest (127 DAS). During the treatment period, the crop in T_1 received a total of 348 mm of irrigation (equivalent to 85% of the cumulative evaporation), while the water-deficit treatment, T_2, received a total of 105 mm of irrigation (equivalent to 25% of the cumulative evaporation). The experiment was laid out as a split-plot design, with the two irrigation regimes as main plots and the six genotypes as sub-plots with four replications.

Measurement of Δ and Superficial Leaf Density

The 3rd or 4th leaves from the apex (upper) and 2nd or 3rd leaves from the soil surface (lower) were sampled separately, from plants harvested from a ground area of 0.75 m^2, at 80, 96, 111 and 127 DAS. Leaf area for each sample (containing about 40 leaflets) was measured by a leaf area meter (LI-COR 3100). The leaves were oven-dried at 80°C for at least 72 h before determining the oven dry weight. Superficial leaf density (ρ_L) was calculated as the ratio of leaf dry weight per leaf area (g m^{-2}).

Carbon isotope composition was analysed in upper and lower leaf samples of the plants harvested for growth analysis at 80 DAS, from three replications. After recording ρ_L, the leaf samples were ground to pass through a 100 μm sieve, and analysed for carbon isotope ratio at the Research School of Biological Sciences, Canberra, as described by Hubick *et al.* (1986).

Analysis of Leaf Proteins

Leaf soluble protein content was determined at 80, 96, 111 and 127 DAS. Fresh leaf samples (about 20 leaflets) from upper and lower positions in the canopy were collected separately from the plants harvested for growth analysis, and their fresh weights and leaf area were recorded. Leaf samples were frozen in liquid nitrogen and stored at –20°C until they were analysed for soluble proteins. For protein analysis, each frozen leaf sample was extracted in 12 mL of Tris–HCl buffer, pH 8.0, and the extract was centrifuged at 27 000 *g* for 15 min. Two aliquots of 1 mL each were sampled from the supernatant for soluble protein analysis using the dye-binding method (Bradford 1976).

Analysis of Rubisco

From the plants harvested at 80 DAS, a separate set of upper and lower leaf samples (about 20 leaflets) were collected for Rubisco analysis. Leaf area of each sample was measured and the samples were frozen in liquid nitrogen until further processing. Frozen leaf samples from 80 DAS were extracted in a known volume of Tris–HCl buffer, pH 8.0, and the extract was centrifuged at 27 000 *g* for 15 min. The supernatant was lyophilised and stored at –20°C for determination of Rubisco at a later stage. Rubisco analysis was performed at the Department of Crop Physiology, University of Agricultural Sciences, Bangalore, using an enzyme-linked immunosorbent assay, using polyclonal antibodies developed against the holoenzyme (Demirevska-Kepova *et al.* 1990).

Statistical Analysis

The data were analysed as a split-plot design with irrigation regimes as main treatments, genotypes as sub-treatments, and leaf positions as sub-sub-treatments, with four replications for ρ_L, Rubisco, and protein estimations and three replications for Δ.

Results

Soluble protein content in leaves ranged from 0.5 to 1.0 g m^{-2} at 80 DAS and increased with time, reaching a peak at 110 DAS, followed by a decline as the crop reached maturity (Fig. 1). There was a marginal increase in soluble protein content of upper leaves under water-deficit conditions (Fig. 1*b*), but this increase was not consistent in all genotypes. Upper leaves (Fig. 1*a* and *b*) tended to have more soluble protein than lower leaves (Fig. 1*c* and *d*). TMV 2-NLM and ICGV 86635 consistently had the highest amount of soluble proteins in upper leaves under normal and water-deficit conditions, while some genotypes (e.g. ICG 221 and ICGV 86707) showed only marginal changes in soluble protein levels under water deficit. Except for TMV 2-NLM, genotypic variation in soluble protein levels was not consistent with time.

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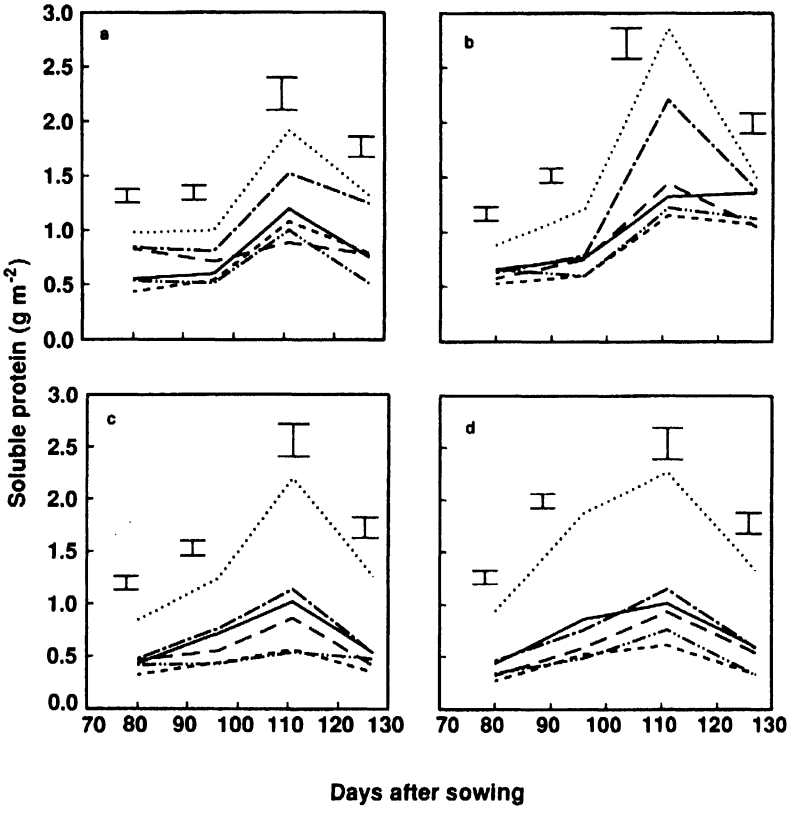

Fig. 1. Changes in soluble proteins with time in leaves from upper (*a* and *b*) and lower (*c* and *d*) positions in the canopy of six groundnut genotypes grown under irrigated (*a* and *c*) and water-deficit (*b* and *d*) conditions at ICRISAT Asia Center, during the 1991–1992 post-rainy season. (——) ICG 476; (– – –) ICG 221; (· · · · ·) TMV 2-NLM; (- - - -) ICGV 86031; (—·—·—) ICGV 86635; (—·—·—) ICGV 86707.

The Rubisco content at 80 DAS ranged from 100 to 300 mg m^{-2} under irrigation (T_1), and from 80 to 450 mg m^{-2} under water deficit (T_2) (Table 1). The upper leaves had significantly greater amounts of Rubisco than the lower leaves under both irrigation and water deficit. Rubisco content increased under water deficit although the extent of the increase varied from 6 to > 42% among genotypes. On an average (pooled over leaf position), TMV 2-NLM, followed by ICGV 86031, had the highest Rubisco content under irrigated and water deficit (Table 1). There was a significantly positive correlation (r^2 = 0.77, P < 0.01) between Rubisco content and soluble protein levels in leaves suggesting that Rubisco constitutes 37% of the leaf's soluble protein pool in groundnut (Fig. 2).

Carbon discrimination values had a range of 3.4‰ (17.5–20.9‰) across all treatment combinations. The mean Δ (of upper and lower leaves) ranged from 18.2 to 20.2‰, representing a significant variability among genotypes (Table 2), with TMV 2-NLM having the lowest and ICG 476 having the highest Δ. There were only marginal changes in Δ, with a trend for reduction under water deficit, but the effect of irrigation regimes on Δ was not significant (Table 3). Leaf positional effect on Δ was significant with upper leaves having consistently lower Δ (range 17.5–19.4‰) than in the lower leaves (range 19–20.9‰) in the canopy (Table 2). Genotype × leaf position interaction was significant for Δ (Table 2 and 3).

Rubisco content and Δ were negatively correlated (r^2 = 0.65, P < 0.01, Fig. 3), suggesting that the genotypes with low Δ had high Rubisco content in leaves. There was also a positive correlation (r^2 = 0.60, P < 0.05) between ρ_L and Rubisco levels in the upper leaves (Fig. 4), although the relationship was not significant in the lower leaves. The overall relationship between ρ_L and Rubisco (r^2 = 0.40, P < 0.05) was not as strong as it was between Rubisco and Δ.

Table 1. Rubisco content (mg m^{-2}) in upper and lower leaves in the canopy of six groundnut genotypes grown under irrigated and water-deficit conditions

Each value is a mean of eight replications. s.e.m. for comparing irrigation regime (I) = 15.9, s.e.m. for comparing genotypes (G) = 20.3, s.e.m. for comparing leaf position (P) = 9.8, s.e.m. for I × G × P interaction = 21.8, CV (%) = 14.4

Genotype	Irrigated		Water deficit	
	Upper	Lower	Upper	Lower
ICG 476	129	102	174	121
ICG 221	154	119	211	80
TMV 2-NLM	283	225	457	268
ICGV 86031	292	134	294	118
ICGV 86635	134	110	211	119
ICGV 86707	226	119	334	141
Mean	203	118	280	142

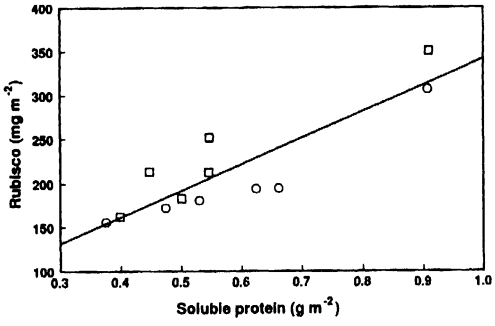

Fig. 2. Relationship between Rubisco content and soluble protein in leaves of six groundnut genotypes grown under irrigated (○) and water-deficit (□) conditions. $y = 42.0 + 299.4x$, $r^2 = 0.77$.

Discussion

Although significant genotypic variation for Δ has been observed in C_3 crops, physiological explanation for the variability vary depending on crop and environment (Hall *et al.* 1993). In the present study, a significant relationship ($r^2 = 0.65$, $P < 0.01$) between Rubisco and Δ in a range of groundnut genotypes (Fig. 3) provides a molecular basis for variation in Δ. Earlier observations in the *Arachis* species (Hubick *et al.* 1986; Wright *et al.* 1988,1993) suggested that variation in photosynthetic capacity per unit leaf area could be largely responsible for genotypic variation in Δ or *W*. A linear relationship between the rate of CO_2 assimilation and the Rubisco levels in leaves has been observed in a number of studies (von Caemmerer and Farquhar 1981; Evans 1986). Carbon isotope discrimination in C_3 plants is dependent on the ratio of internal to ambient CO_2 pressures (p_i/p_a) which is controlled by the rate of diffusion of CO_2 from the atmosphere to the sites of carboxylation (leaf conductance) relative to the ability of the leaf to assimilate CO_2 during photosynthesis (assimilation rate) (Farquhar *et al.* 1982). In the present study, it is likely that the increase in the assimilation rate caused by an increase in photosynthetic capacity is the main cause of a decrease in Δ across genotypes. In fact, it is likely that the increase in capacity associated with an increase in Rubisco content, may have been accompanied by a decrease in diffusion resistance, as the range in Δ was only 3‰ despite Rubisco content tripling across this range (Fig. 3). If the 7‰ decrease from the potential maximum of *ca* 27 to 20‰ occurs with a Rubisco content of 100 mg m^{-2}, then an increase in Rubisco content to 300 mg m^{-2} would cause Δ to drop by about (7 × 300/100 =) 21 to 6‰ if diffusive resistance were not to change. It seems then that assimilation rate and total conductance probably changed largely in parallel, as commonly observed for the stomatal component (Wong *et al.* 1979), and for the overall conductance component (von Caemmerer and Evans 1991), but with slightly greater changes in capacity and assimilation rate than in total conductance. This is in contrast to the results of, for example, Ehleringer (1990), who found conductance changes dominating in *Phaseolus vulgaris* cultivars. In a study of 14 wheat genotypes, Condon *et al.* (1990) found that variation in Δ was roughly equally ascribable to changes in capacity and conductance.

The soluble protein levels in leaves, varied significantly among genotypes, with leaf position in the canopy, and also with time through the season (Fig. 1), suggesting spatial and temporal variation in leaf proteins. Rubisco constituted about 37% of the soluble protein in groundnut genotypes tested in the present study (Fig. 2). The proportion of Rubisco in the soluble protein pool in groundnut (37%) is comparable with that observed in other C_3 crops (Evans 1989; Sinclair and Horie 1989; Quick *et al.* 1991). Significant correlations between specific leaf nitrogen and Δ (Wright *et al.* 1993), and between leaf nitrogen and

Table 2. Carbon isotope discrimination (‰) in upper and lower leaves in the canopy of six groundnut genotypes grown under irrigated and water-deficit conditions

Each value is a mean of three replications. s.e.m. for comparing irrigation regime (I) = 0.06, s.e.m. for comparing genotypes (G) = 0.12, s.e.m. for comparing leaf position (P) = 0.04, s.e.m. for I × G × P interaction = 0.18, CV (%) = 1.1

Genotype	Irrigated		Water deficit	
	Upper	Lower	Upper	Lower
ICG 476	19.4	20.9	19.2	20.3
ICG 221	18.0	19.9	18.5	20.0
TMV 2-NLM	17.5	19.0	17.2	18.8
ICGV 86031	17.9	19.5	18.2	20.1
ICGV 86635	18.2	20.4	17.9	20.4
ICGV 86707	18.2	19.9	18.0	19.5
Mean	18.2	19.9	18.2	19.9

Table 3. Analysis of mean sum of squares of Δ and Rubisco content for six groundnut genotypes as influenced by leaf position, irrigation regime and genotype

** significant at $P = 0.01$

Source of variation	df	Mean sum of squares	
		Δ	Rubisco
Replication	2	2.587	2137.4
Irrigation (I)	1	0.055	504.4
Genotype (G)	5	4.24**	769.4**
I × G	5	0.339	37.3
leaf position (P)	1	52.360**	3642.9**
I × P	1	0.045	59.2
G × P	5	0.335**	8.2
I × G × P	5	0.081	10.3
Total	71		

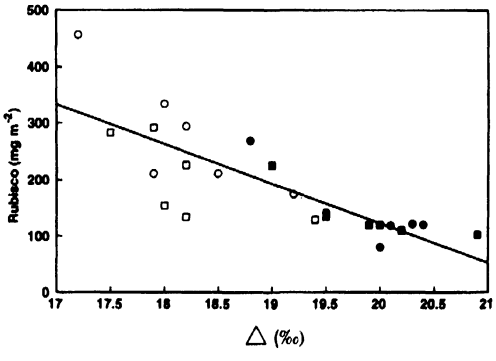

Fig. 3. Relationship between Rubisco content and Δ in upper (□, ○) and lower (●, ■) leaves of six groundnut genotypes grown under irrigated (□, ■) and water-deficit (○, ●) conditions. $y = 1528 - 70x$, $r^2 = 0.65$.

photosynthetic capacity regardless of the age of the leaf (Connor *et al.* 1993), suggest a linkage between leaf protein content and Δ.

Unlike many studies (e.g. in wheat (Farquhar and Richards 1984), in groundnut (Wright *et al.* 1988, 1993, 1994) and in cowpea (Hall *et al.* 1992)), the effect of water deficit on Δ observed in the present study was small. Perhaps a greater effect would have been observed in leaf sugars (Brugnoli *et al.* 1988), or in the most recently formed leaves. However, it should be noted that in the studies of Hubick *et al.* (1986), the effects of withholding water on Δ of groundnut were small. Furthermore, Wong *et al.* (1985) found virtually no effect of water stress on the intercellular CO_2 concentration. The results may depend on the speed of soil drying.

It is interesting to note that Rubisco levels per unit leaf area increased under water deficit, although this effect was not significant (Table 1). This is similar to the effect of increased resistance to soil penetration under water deficit reported by Masle and Farquhar (1988) in a wheat cultivar. Cell expansion is known to be very sensitive to water deficit (Slatyer 1955; Allen *et al.* 1976), thus, mild water deficit may have influenced leaf thickness by increasing numbers of chlorenchyma cells and chloroplasts per unit leaf surface area (Nobel 1991).

Significant relationships between W, Δ and specific leaf area, SLA (inverse of ρ_L) (Wright *et al.* 1994), and negligible genotype × environment interaction for Δ and SLA in groundnut (Nageswara Rao and Wright 1994), suggested the possibility of using SLA as a surrogate for Δ in selecting genotypes with high W. However, the extent to which Δ may be used as a screening tool in breeding programs depends on consistency in ranking of genotypes in a wide range of environments, which is influenced by various factors including tissue sampling methodology. In

the present study, significant leaf positional effects on Δ and Rubisco within treatments were evident (Tables 2 and 3) emphasising the importance of leaf sampling in comparative screening studies using Δ or leaf thickness as a screening tool. High Δ and low Rubisco in lower leaves might be due to leaf aging or to low irradiance. A decline in photosynthetic capacity and activity of Rubisco with increasing leaf age has been shown (Evans 1986; Connor *et al.* 1993). The significant genotype × leaf position interaction for Δ (Table 3), however, means that the leaf position in the canopy can significantly influence the selection of genotypes for *W* based on Δ or ρ_L. It is to be noted that the leaves closer to the soil contain carbon fixed when the leaves are partly shaded, and as the canopy grows they are fully shaded leading to several changes in metabolic activities. Indeed for selection purposes in some crops like wheat, early formed leaves are recommended (Condon *et al.* 1990). Spatial variations in Δ and Rubisco observed in the present study highlight the importance of leaf position in selecting for *W*, using leaf traits in groundnut. To avoid confounding effects due to age and shade on Δ and Rubisco, it is suggested that selection for *W* based on Δ or ρ_L should be practiced on upper leaves in groundnut.

The results from this study also suggest that most of the variation in Δ (and thus *W*) in groundnut genotypes can be ascribed to variation in Rubisco content in the leaves. It was interesting to note that a physical parameter like ρ_L was correlated with a biochemical component of leaves, i.e. Rubisco content, although the relationship between ρ_L and Rubisco was not as strong as it was between Δ and Rubisco.

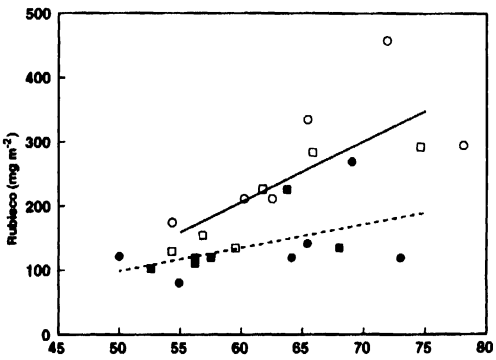

Fig. 4. Relationship between Rubisco content and superficial leaf density (ρ_L) in upper (□, ○) and lower (●, ■) leaves of six groundnut genotypes grown under irrigated (□, ■) and water-deficit (○, ●) conditions. For upper leaves, $y = -361 + 9.4x$, $r^2 = 0.60$. For lower leaves, $y = -82 + 3.6x$, $r^2 = 0.24$.

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