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RESEARCH PAPER

Differential adaptation of two varieties of common bean to abiotic stress

II. Acclimation of photosynthesis

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Abstract

The photosynthetic characteristics of two contrasting varieties of common bean (*Phaseolus vulgaris*) have been determined. These varieties, Arroz and Orfeo, differ in their productivity under stress conditions, resistance to drought stress, and have distinctly different stomatal behaviour. When grown under conditions of high irradiance and high temperature, both varieties displayed evidence of photosynthetic acclimation at the chloroplast level—there was an increase in chlorophyll *a*/*b* ratio, a decreased content of Lhcb proteins, and an increased xanthophyll cycle pool size. Both varieties also showed reduced chlorophyll content on a leaf area basis and a decrease in leaf area. Both varieties showed an increase in leaf thickness but only Arroz showed the characteristic elongated palisade cells in the high light-grown plants; Orfeo instead had a larger number of smaller, rounded cells. Differences were found in stomatal development: whereas Arroz showed very little change in stomatal density, Orfeo exhibited a large increase, particularly on the upper leaf surface. It is suggested that these differences in leaf cell structure and stomatal density give rise to altered rates of photosynthesis and stomatal conductance. Whereas, Arroz had the same photosynthetic rate in plants grown at both low and high irradiance, Orfeo showed a higher photosynthetic capacity at high irradiance. It is suggested that the higher yield of Orfeo compared with Arroz under stress conditions can be explained, in part, by these cellular differences.

Key words: Abiotic stress, acclimation, common bean (*Phaseolus vulgaris*), drought, photosynthesis, stomata.

Introduction

The degree of tolerance of plants to environmental stress varies greatly not only between species but in different varieties of the same species. A thorough understanding of the physiological basis of such differences in stress tolerance could be used to select or create new varieties of crops that have increased productivity under such conditions. Two contrasting varieties of common bean, Orfeo and Arroz, have been identified that have different yield responses to stress: Orfeo, the more stress-tolerant variety, has been shown to have better water retention, a lowered rate of abscission of flowers, and less photoinhibition under drought conditions, and was found to exert greater dynamic control over stomatal opening (Lizana *et al*., 2006). When grown under the high light and high temperature 'stress' conditions that resemble those found in the field, Orfeo had a higher photosynthetic rate than the stress-sensitive Arroz, whereas under 'control' conditions of low light and low temperature their photosynthetic rates were identical. One explanation of the difference in photosynthetic rate is that photoacclimation of photosynthesis (i.e. the optimization of the composition of the leaf for photosynthesis in high irradiance) might be better expressed in Orfeo compared with Arroz.

Photoacclimation is a complex array of changes occurring in the leaf (Björkman, 1981; Anderson *et al.*, 1995; Bailey *et al*., 2001; Walters *et al*., 2003) and can be

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considered to consist of leaf level acclimation and chloroplast level acclimation (Murchie and Horton, 1997). Chloroplast level acclimation refers to the differences in content of thylakoid proteins, pigments, Calvin cycle enzymes, etc, on a per chloroplast basis (Anderson *et al*., 1995; Murchie and Horton, 1998). Parameters such as the chlorophyll *a*/*b* (Chl a/b) ratio, the PSII/PSI ratio, or P_{max} per unit chlorophyll are indicative of chloroplast level acclimation. Leaf level acclimation refers to the markedly different anatomy of high- and low-light leaves: a generalized picture of 'sun-type' morphology would show thicker leaves with more, columnar mesophyll cells (Leech *et al*., 1980; Mullet, 1988; Sims and Pearcy, 1992; Oguchi *et al*., 2003; Yano and Terashima, 2004). Parameters such as total numbers of chloroplasts, total chlorophyll, protein, or Rubisco per unit leaf area are strongly influenced by leaf level acclimation. Although less widely studied, leaf level acclimation is associated with changes in stomatal numbers on the leaf surface(s), with an increase in both stomatal density and stomatal index occurring in high light-grown leaves (Lake *et al*., 2002; Schlüter *et al.*, 2003).

Leaf level and chloroplast level acclimation are differently regulated and can be separated experimentally. Leaf level acclimation seems to be largely controlled by signals perceived and generated in mature leaves and transduced to newly developing leaves, whereas chloroplast level acclimation is regulated by ambient events (Yano and Terashima, 2001; Oguchi *et al*., 2003). Leaf level acclimation is determined at an unknown point early on in leaf expansion, is usually not reversible, and cannot be induced in leaves grown under low light when they are transferred to high light (Yano and Terashima, 2004; Murchie *et al*., 2005). Therefore, when plants are transferred from low to high irradiance, generally, only chloroplast level acclimation occurs, the full extent of acclimation only being observed by comparing plants grown under different irradiances.

In this paper, chloroplast level and leaf level acclimation have been compared in Orfeo and Arroz for plants grown under low light and high light in order to test the hypothesis that the extent of photoacclimation determines the differential photosynthetic rate and yield of these varieties under stress conditions. It is shown that, although chloroplast level acclimation is almost identical in both varieties, there are significant differences at the leaf level, particularly in stomatal number and leaf cell structure. It is suggested that these latter differences can explain, in part, the contrasting degrees of stress tolerance in these varieties.

Materials and methods

Plant material and growth conditions

Two varieties of common bean (*Phaseolus vulgaris*), Orfeo and Arroz, were used. Plants were germinated and grown on M2 commercial compost (Levington's) under a 12 h photoperiod. Material was maintained under standard conditions of either low light (LL) (300 μmol m^{-2} s^{-1}/22–25 °C) or high light (HL) (1000 μmol m^{-2} s^{-1}/32–35 °C). In some experiments, where stated in the text, plants were also grown under LL but at 32–35 °C and HL at 22–25 °C.

Photosynthesis and chlorophyll fluorescence measurements

Photosynthetic gas exchange was measured using a Li-Cor (Lincoln, NB, USA) 6400 portable photosynthesis system with a fluorometer attachment (6400-02) which provided irradiance by means of an array of red and blue light-emitting diodes. Measurements were made in the growth room, using ambient humidity (40–60% RH).

Determination of stomatal numbers

Mature leaves were removed from the plant and the stomatal density determined, as described in Salisbury (1927), from the adaxial and abaxial surfaces of the leaf using the dental rubber impression technique (Weyers and Johansen, 1985). At least four assays were carried out on random areas of mature leaves (a field of view was routinely taken at a magnification of ×100) from at least six individual plants for each growth irradiance. Results represent mean ±standard error (n >24).

Microscopy

Leaf segments, ~1 mm wide, were cut in water with a new razor blade from a freshly excised leaf and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for a minimum of 24 h. These were washed in two changes of 0.1 M phosphate buffer for 1 h and then dehydrated through graded alcohol solutions (70%, 90%, 100%) for a minimum of 1 h per solution. Tissue was then infiltrated in JB-4® solution A plus catalyst (as per the manufacturer's instructions) overnight at 4 °C and then embedded in fresh JB-4® solution A with catalyst (100 ml) and 0.8 ml solution B. Polymerization was overnight at 4 °C. Unpolymerized resin was removed from the block by rinsing briefly in 70% alcohol and air drying. Sections, 4 μm thick, cut using an LKB Historange microtome and a glass knife were collected over water and stained in 0.05% toluidine blue in acetate buffer pH 4.4 for 2 min and washed in distilled water for 2 min, dried on a hotplate, and mounted in DPX. Mounted sections were used for measurements of leaf thickness and analysis of leaf structure using an Olympus BX51 microscope with a digital camera attachment at a magnification of ×40–100 depending on the sample.

HPLC analysis of leaf carotenoid content

Carotenoids were analysed essentially using the technique of Farber *et al*. (1997). Briefly leaf discs were taken, flash frozen in liquid N_2, and extracted by grinding into 100% acetone. The pigment extract was left in the dark for 30 min before being centrifuged at 15 700 *g* for 5 min to remove any cell debris. Samples were loaded and run on a Dionex HPLC system. Pigments were separated using a LiChroCART® RP-18 (Merck) column and the peaks detected using a Dionex PDA-100 photodiode array detector set to record between 230 and 800 nm. Data analysis was carried out using the Chromelian HPLC software (Dionex).

Electrophoresis and western blot analysis

Electrophoresis and western blot analysis to determine the content of Lhcb proteins was performed on thylakoids as described previously (Ruban *et al*., 2003).

Results

Figure 1 shows the irradiance dependency of photosynthetic rate, in ambient (A) and saturating CO_2 (B) for Arroz and Orfeo grown under control conditions of low light and

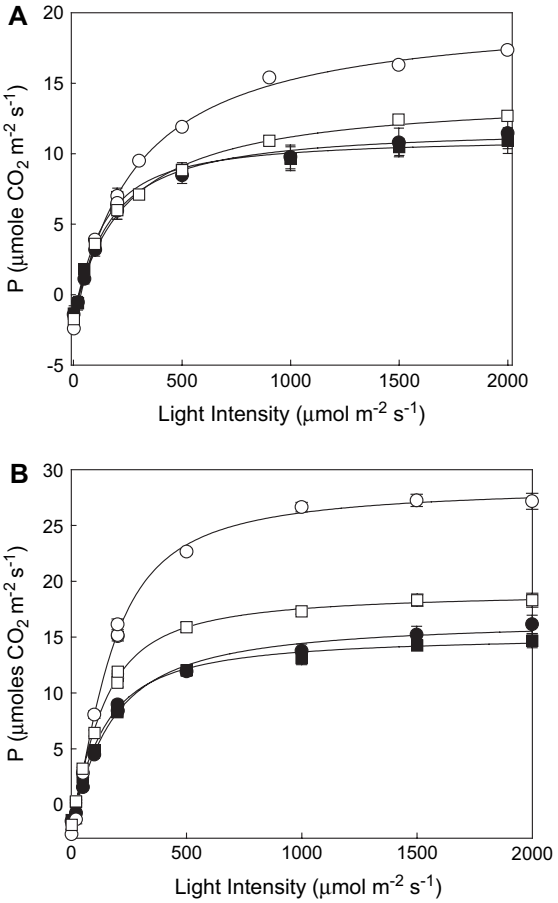


Fig. 1. Photosynthetic rate (*P*) at different light intensities for leaves grown under low (LL) and high light (HL) conditions measured at (A) 350 μl l^{-1} CO_2 and (B) 900 μl l^{-1} CO_2. Plants were grown under low light (300 μmol m^{-2} s^{-1}/20–25 °C) or high light (1000 μmol m^{-2} s^{-1}/32–35 °C). Circles, Orfeo; squares, Arroz; filled symbols, LL; open symbols, HL.

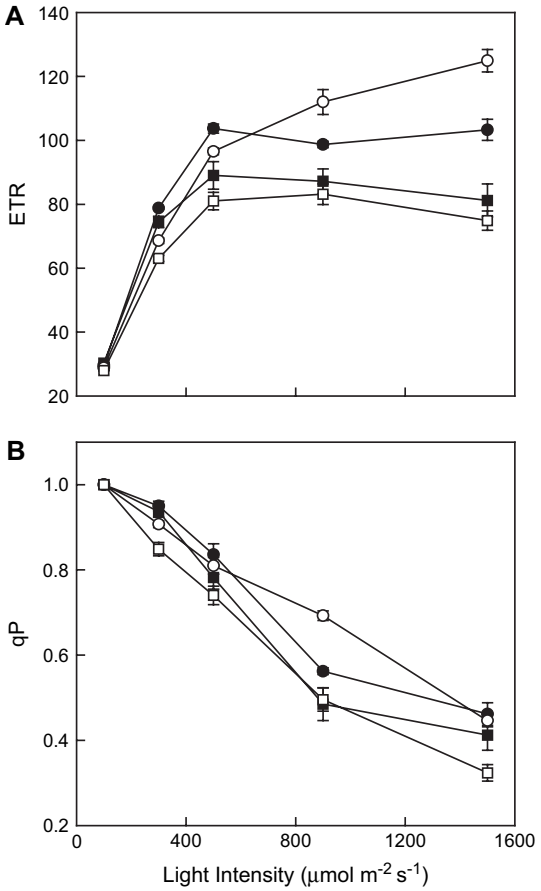


Fig. 2. Fluorescence parameters at different light intensities for leaves grown under low (LL) and high light (HL) conditions measured at ambient CO_2. (A) Calculated electron transport rate (ETR); (B) *q*P, photochemical quenching. Plants were illuminated at 350 μl l^{-1} CO_2. Growth conditions were as described in Fig. 1. Circles, Orfeo; squares, Arroz; filled symbols, LL; open symbols, HL.

temperature, compared with high light and high temperature. Under control conditions, both varieties exhibited very similar responses. However, Arroz and Orfeo were very different when grown under high-light conditions – the light-saturated rate was much higher in Orfeo than Arroz. Indeed, the difference between HL and LL plants was very small for Arroz. Similar results were obtained at both ambient (Fig. 1A) and saturating CO_2 (Fig. 1B). It is important to note that no differences in quantum yield between Arroz and Orfeo were observed under limiting light, indicating that the lower photosynthetic rate in Arroz was not due to differences in extent of photoinhibition. In confirmation of this, under these conditions, the dark-adapted F_v/F_m was 0.80 and 0.79 for Orfeo and Arroz, respectively (not shown).

Analysis of chlorophyll fluorescence confirmed the differences in photosynthetic rate between Arroz and Orfeo (Fig. 2). Estimated electron transport rates showed the large difference between Orfeo and Arroz when grown under HL, with a much smaller difference observed for LL plants. Under HL conditions, the latter variety saturated its electron transport rate at a light intensity of about 400 μmol m^{-2} s^{-1}. This does not happen in Orfeo where the electron transport showed a steady increase until 1500 μmol m^{-2} s^{-1}. The excitation pressure on PSII was significantly less in HL-grown Orfeo than in HL-grown Arroz, as deduced from the higher values of *q*P. For LL plants, there were only small differences in *q*P between Arroz and Orfeo.

The data in Figs 1 and 2 suggest that Arroz was not exhibiting the same extent of photoacclimation of photosynthesis as Orfeo. However, comparison between LL and HL leaves showed that there was a large number of differences in both varieties. First, the leaves of HL plants were smaller and thicker with reduced chlorophyll content (Table 1). Chloroplast level acclimation was investigated—Table 1 shows that, in both Arroz and Orfeo, the Chl a/b ratio increased from values of ~3.4, typical for LL-grown plants, to ~4.0, as found in many HL-grown plants. This change indicates a decrease in the amounts of light-harvesting complexes relative to reaction centres, i.e.

Table 1. *Parameters of leaf morphology and composition for plants under low-light (LL) and high-light (HL) conditions*

Growth conditions were as described in Fig. 1. Data were taken from mature fully expanded leaves and are the averages ±standard error of at least three replicate assays from at least six separate plants per batch.

Variety		Leaf area (cm^2)	Leaf thickness (μm)	Rubisco content (g m^{-2})	[Chl] (μg cm^{-2})	Chl a/b
Orfeo	LL	54.9±7.9	226±4	2.56±0.20	56.3±4.2	3.41±0.05
	HL	29.9±3.7	331±4	2.90±0.21	27.1±1.6	3.96±0.06
Arroz	LL	44.9±5.5	242±4	2.34±0.21	58.8±2.1	3.35±0.04
	HL	21.1±2.3	296±3	2.61±0.20	26.1±2.8	4.01±0.06

a decrease in antenna size. Direct quantification of the content of the main light-harvesting proteins, Lhcb1 and Lhcb2, showed that they both decreased relative to PSII reaction centre content by ~60% and 30%, respectively, to the same extent in both varieties (Fig. 3). Finally, the changes in carotenoid composition were also the same; in HL plants the carotenoid/chlorophyll ratio was higher and the proportion of carotenoid found as the xanthophyll-cycle carotenoids increased from 16–18% in LL, plants to ~30% in HL plants (Table 2). The de-epoxidation of the xanthophyll cycle pool was close to zero in LL, but increased to 30% in Arroz and 40% in Orfeo in HL; this indicates a significant extent of light saturation under these conditions.

Although both Arroz and Orfeo had thicker leaves under HL conditions, microscopical analysis of transverse leaf sections revealed clear differences in their cell structure (Fig. 4A). In LL, both had a rather similar structure—the leaf was predominantly spongy mesophyll cells, with a very ill-defined palisade layer of one or two cells below the adaxial epidermis. The stomata and air spaces are located towards the abaxial surfaces. In HL plants, the section of Arroz showed a cell organization typical of a 'sun' leaf—a layer of elongated columnar palisade cells, about two cells thick (Fig. 4B, C), was found at the adaxial surface, above the spongy mesophyll cells. There were occasional air spaces, which correspond to substomatal cavities, at the adaxial surface but most were again at the abaxial surface (Fig. 4D, E). The leaves of HL Orfeo were very different—at the adaxial surface there was a layer of a large number of rounded cells, so that the palisade layer was ill-defined, as in the LL plants, but much thicker. The depth of this layer was about the same as in Arroz, but the number of cells was increased 3-fold (Fig. 4B, C). It was also found that the adaxial surface had a greatly increased number of air spaces, equal to the frequency on the abaxial surface (Fig. 4D, E).

The data in Fig. 4 indicated differences in the stomatal frequency in Orfeo and Arroz. Therefore, a detailed analysis was undertaken, and the results are shown in Figs 5 and 6. Under LL conditions, there was a high frequency of stomata on the abaxial surface and a low frequency on the adaxial surface in both Arroz and Orfeo (Fig. 5). Quantitative analysis confirmed these observations, and also showed that the frequency of stomata on both the abaxial and adaxial surfaces in LL plants was slightly higher in Arroz than in Orfeo (Fig. 6).

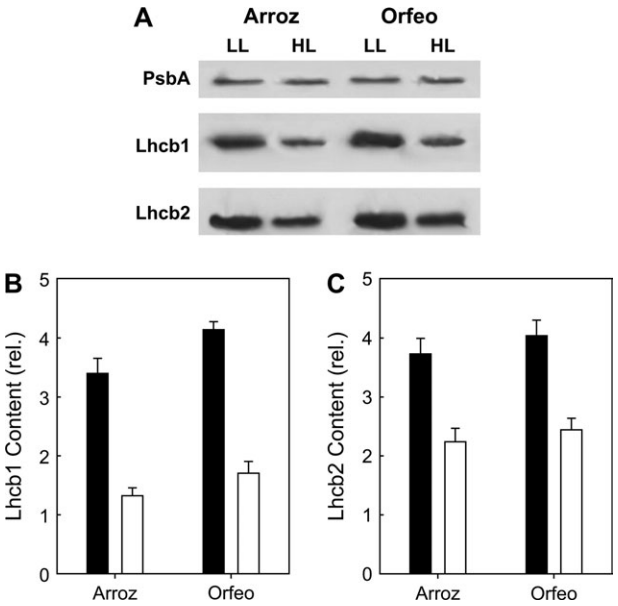


Fig. 3. Contents of Lhcb1 and Lhcb2 proteins in beans grown under low light (LL) and high light (HL) conditions. (A) Western blots using antibodies to Lhcb1, Lhcb2, and PsbA. (B, C) Results of densitometric estimations of the contents of Lhcb1 and Lhcb2 normalized to the content of PsbA, for LL (black) and HL (white) plants. Thylakoid proteins were run on 15% SDS-PAGE, with loading normalized on PSII content reaction centre protein (PsbA) determined using trial blots (for LL plants this was ~3 μg chl $lane^{-1}$). Growth conditions were as described in Fig. 1. Measurements were taken from at least four individual blots. Data represents mean ±standard error (n >4).

The frequency of stomata showed significant changes in HL conditions compared with LL and, moreover, the responses of Orfeo and Arroz were very different. Comparing the abaxial surfaces, there was little change in Arroz (Fig. 5), but in Orfeo there was a clear increase in the density of stomata, almost to a state of maximum possible differentiation of epidermal cells into stomata. Quantitative analysis showed that the frequency of stomata on the abaxial surface of Orfeo increased by almost 2-fold in HL compared with LL, by contrast to that observed in Arroz, with no significant differences between HL and LL plants (Fig. 6A).

Unexpectedly, changes in stomatal frequency were also observed on the adaxial leaf surfaces, particularly in Orfeo. In Arroz, adaxial stomata appeared to be more frequent in HL than LL, but still much less than on the abaxial

Table 2. *Carotenoid composition of leaves grown under low-light (LL) and high-light (HL) conditions*

Neo, neoxanthin; Vio, violaxanthin; Anth, antheraxanthin; Lut, lutein; Zea, zeatin; β-car, β-carotene, XC, vio+anth+zea; Car/Chl, molar ratio of total carotenoid to total chlorophyll; DEPS, de-epoxidation state (zea+½anth)/XC. Growth conditions were as described in Fig. 1. Data are the averages ±standard error of at least three replicate assays from at least six separate plants per batch.

Variety		Neo (%)	Vio (%)	Anth (%)	Lut (%)	Zea (%)	β-car (%)	XC (%)	Car/Chl	DEPS
Orfeo	LL	13.5±0.2	15.8±0.3	1.4±0.2	39.5±0.6	ND	28.4±0.4	18.6±0.6	0.39±0.01	3.7±0.5
	HL	10.1±0.5	14.4±0.8	6.1±0.9	32.0±0.7	9.0±1.0	31.1±1.5	29.5±1.5	0.51±0.03	40.2±2.6
Arroz	LL	13.1±0.2	17.2±0.6	0.9±0.1	41.1±0.7	ND	29.1±0.9	16.7±0.2	0.36±0.01	2.7±0.2
	HL	10.1±0.8	18.8±1.8	4.3±0.4	30.1±1.5	9.0±2.2	29.5±0.9	32.1±3.5	0.47±0.03	32.8±4.3

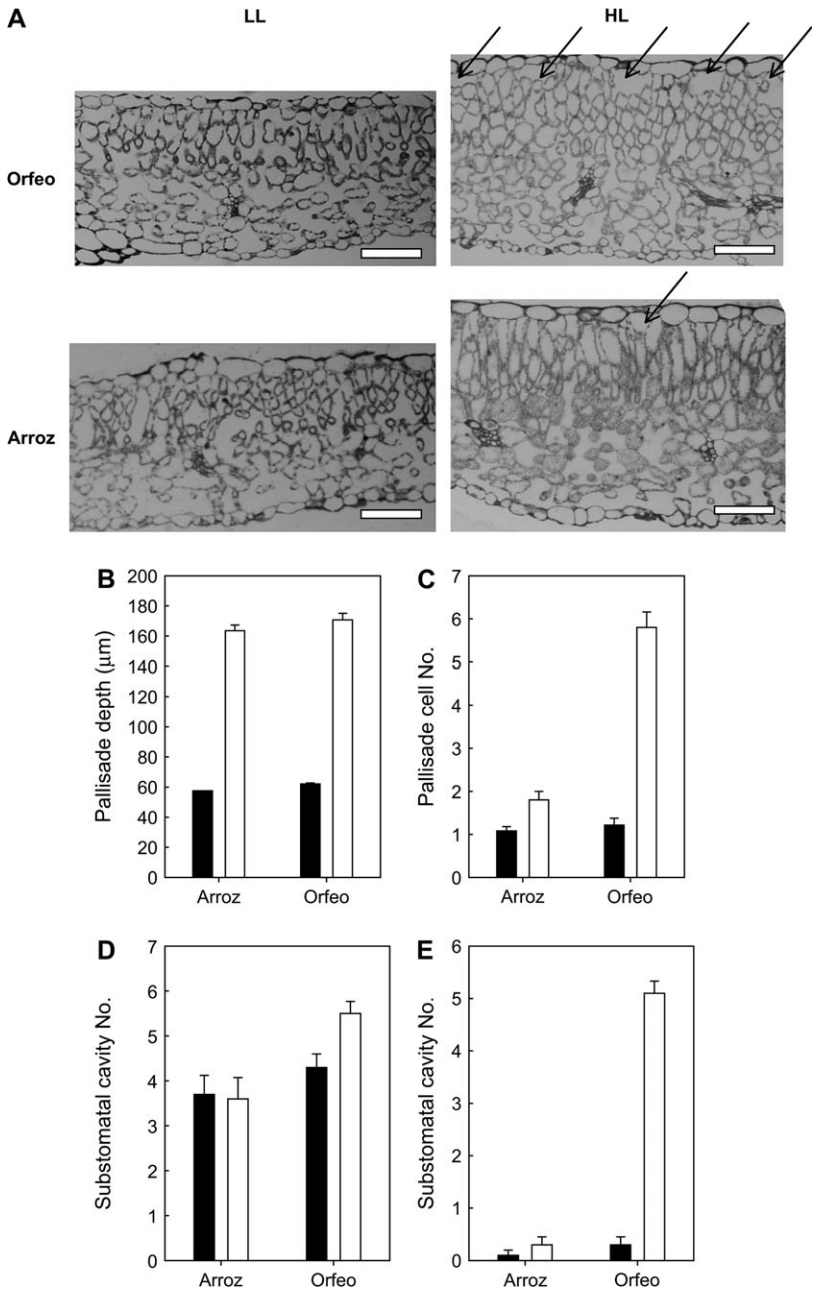


Fig. 4. Leaf cell organization in LL and HL plants. Growth conditions were as in Fig. 1. (A) Micrographs of sections from fully expanded second leaf, stained with toluidine blue. Arrows indicate substomatal cavities; white bars show scale=100 μM. (B) Depth of palisade layer; (C) number of palisade cells above the mesophyll layer; (D) number of abaxial substomatal cavities; (E) number of adaxial substomatal cavities. Black columns, low light; white columns, high light. Measurements were taken from at least six plants and results represent mean ±standard error (n >24).

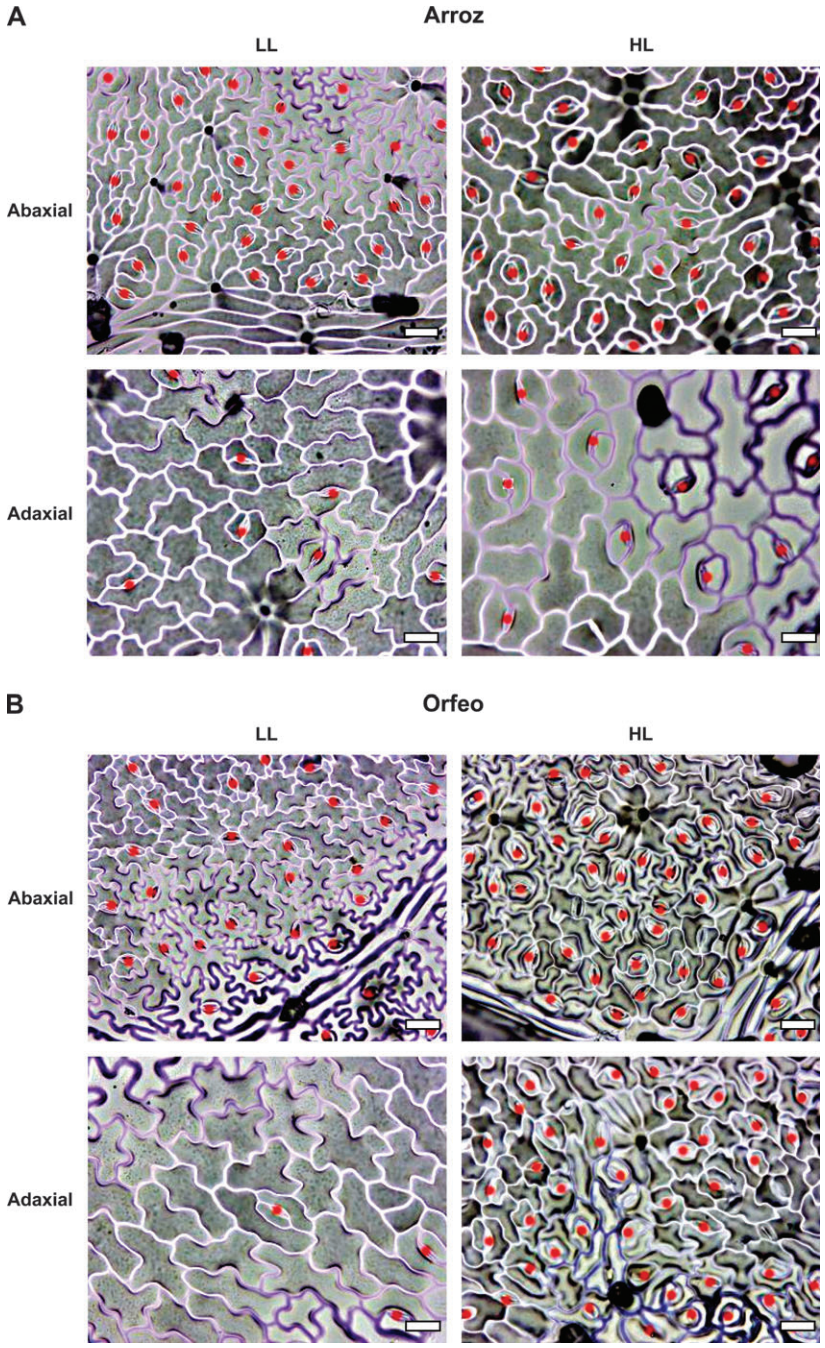


Fig. 5. Abaxial and adaxial surfaces of low light (LL) and high light (HL) leaves of Arroz (A) and Orfeo (B). Stomata have been coloured red for clarity. Growth conditions were as described in Fig. 1. (A) LL, Arroz abaxial surface; HL, Arroz abaxial surface; LL, Arroz adaxial surface; HL, Arroz adaxial surface. (B) LL, Orfeo abaxial surface; HL, Orfeo abaxial surface; LL, Orfeo adaxial surface; HL, Orfeo adaxial surface. White bars show scale=50 μM.

surface (Fig. 5). By contrast, in Orfeo grown under HL, large numbers of stomata were found on the adaxial surface. This was confirmed by the data in Fig. 6B, which shows that the frequencies of stomata on the adaxial surface in HL increased by about 3-fold in Arroz but by about 20-fold in Orfeo. Thus, in Orfeo the frequency of stomata on the adaxial surface was about the same as on the abaxial surface.

All of the data presented so far have analysed plants grown in low light (LL) at low temperature, whilst plants grown in high light (HL) were grown at high temperature. These conditions were chosen to represent a 'control' condition without any stress and a 'stressed condition' simulating that frequently found in the field. It was important to ascertain whether the characteristics of Orfeo leaves in the HL conditions were due to the effect of HL or of the

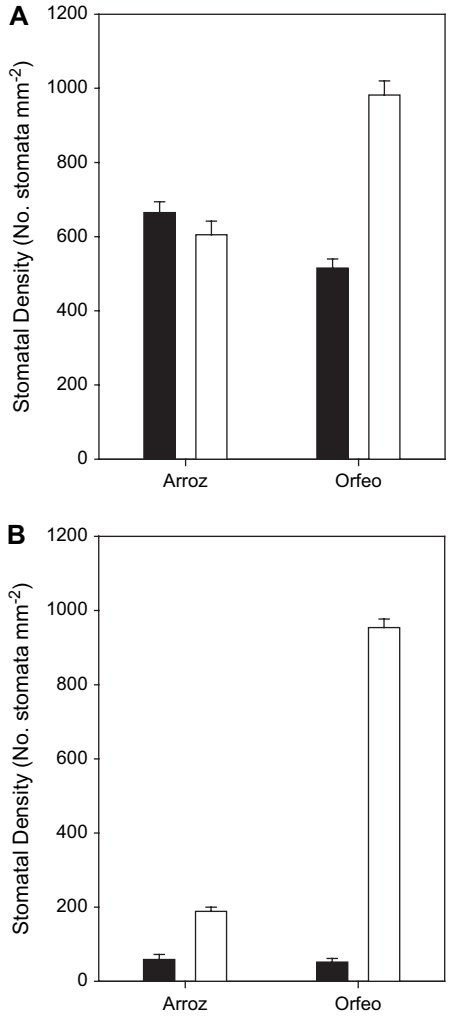


Fig. 6. Stomatal density (no. mm^{-2}) on the abaxial (A) and adaxial (B) surface of low light-(black columns) and high light-grown (white columns) leaves of Arroz and Orfeo. Measurements were taken from images of the type shown in Fig. 5, from different portions of mature leaves from at least six individual plants. Results represent mean ±standard error (n >24).

temperature change, or a combination of both. Therefore, an experiment was carried out in which temperature and irradiance were independently varied (Figs 7, 8). In terms of leaf cell organization, it is clear that the increased leaf thickness (Fig. 7A) and the increased depth of the palisade layer (Fig. 7B) were almost totally dependent upon the increase in irradiance, for both Arroz and Orfeo. In Arroz, the small increase in palisade cell number was dependent only on light (Fig. 7C). For Orfeo, the palisade cell number responded to both light and temperature. Thus, there was a difference between the number of palisade cells both in LL/LT compared with LL/HT, and between HL/LT and HL/HT. However, the largest change was light-dependent; at both LT and HT the number of cells increased by over 2–3-fold in high light compared with low light.

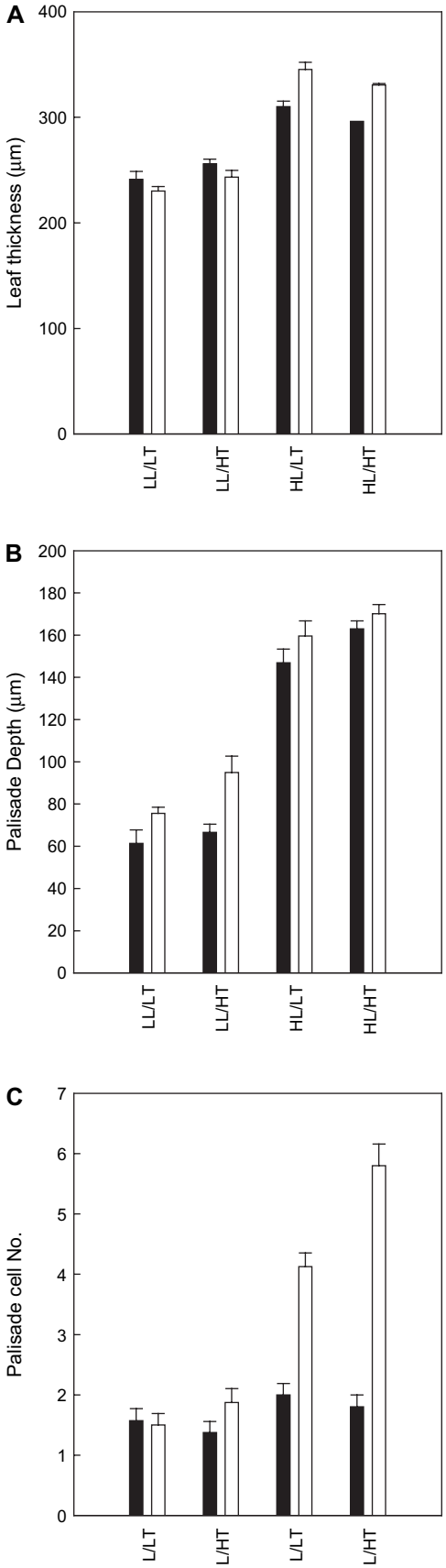
The abaxial stomatal frequency in Arroz was affected by the increase in irradiance, in which a slightly larger increase was found at LT than at HT (Fig. 8A). No differences in stomatal frequency were observed in LL/LT- and LL/HT-grown Arroz; however, at high light, a higher growth temperature resulted in a slightly decreased stomatal frequency in this variety. In Orfeo, the increase in stomata frequency on the abaxial surface was irradiance dependent, although the response was greater at higher temperature (Fig. 8A). Here, the increase in temperature resulted in a slight *increase* in abaxial stomatal frequency.

The stomatal frequency on the adaxial surface was also principally responding to light, although clear effects of temperature were again found (Fig. 8B). In Orfeo, at LT, the increase in irradiance resulted in about a 6-fold increase, compared with around 20-fold at HT. In Arroz, at LT there was an increase in stomatal frequency of about 2-fold but, interestingly, at HT the stomatal frequency was the same in low light compared with high light. In this variety, at low light, an increase in temperature resulted in a 2-fold increase in frequency, whereas at high light, the increase in temperature had no effect. By contrast, in Orfeo, the increase in temperature resulted in increases in stomatal frequency in both low and high light.

Experiments were carried out to attempt to explain the higher P_{max} of Orfeo compared with Arroz that was observed only in HL plants. Increases in Rubisco are commonly associated with photoacclimation. However, in both Arroz and Orfeo, the levels of Rubisco protein only increased by ~10% in HL compared with LL (Table 1). Moreover, there was little difference in the Rubisco contents of Arroz and Orfeo. A/C_i curves for Arroz and Orfeo were consistent with this (Fig. 9). For both LL plants and HL plants the initial slopes of the A/C_i curves were identical, indicating equal Rubisco activities. However, it is also clear that the CO_2-saturated rate of photosynthesis was different between Orfeo and Arroz, but only for the HL-grown plants. As this rate is much higher in Orfeo it indicates an increase in RuBP regeneration capacity for this variety compared with Arroz, most likely due to an increase in its electron transport capacity. This is consistent with the estimates of electron transport rate shown in Fig. 2.

The absence of any difference in slope of the A/C_i curve at first sight is inconsistent with the increased P_{max} of Orfeo at ambient CO_2. However, it was found that when recording the measurements shown in Fig. 1 at saturating irradiance at ambient CO_2, the C_i of HL Orfeo was 260 μl l^{-1} and that of HL Arroz was 230 μl l^{-1}. Examination of Fig. 9 (inset) indicates that this would give photosynthetic rates of ~15 and 12 μmol CO_2 m^{-2} s^{-1}, respectively. The stomatal conductance of Orfeo was correspondingly ~50% higher than that of Arroz under these conditions.

The data in Figs 5 and 8 showed that there was a difference in stomatal frequency between HL Arroz and HL Orfeo which was most clear on the adaxial surface. In the data shown in Fig. 1, leaves were assayed when illuminated on the adaxial leaf surface and, therefore, the stomatal



conductance and photosynthetic rate may have been influenced by this difference in stomatal frequency. Therefore, measurements of HL plants were made in which leaves were illuminated on the abaxial surface (Fig. 10). At ambient CO_2 there was no difference in photosynthetic rate—very low rates were obtained for both Orfeo and Arroz. At saturating CO_2, the rates for both Arroz and Orfeo increased up to values similar to those shown in Fig. 1, and hence the difference between them was restored.

Discussion

Arroz and Orfeo are two contrasting varieties of bean which have different productivities and sensitivities to stress when analysed in a range of field conditions. In Lizana *et al*. (2006) it was shown that, in particular, Arroz was much more sensitive to water stress, and a higher photosynthetic capacity was observed in Orfeo grown under stress conditions (high light+high temperature+drought). Differences in the dynamics of stomatal conductance were also found, which were consistent with Orfeo being better able to manage its water status under stress conditions.

In this paper, the response of Orfeo and Arroz to stress conditions has been characterized in terms of the photoacclimation of the chloroplast and leaf. It was shown that major features of chloroplast composition were the same in Arroz and Orfeo, in both control LL conditions and stress HL conditions. The adjustments in light-harvesting protein and pigment content associated with the photoacclimation of photosynthesis were observed for both varieties. In both cases, there was a reduction in chlorophyll content, a decrease in leaf size, and an increase in leaf thickness, all typical of photoacclimation to increased irradiance (Bjorkman, 1981). Interestingly, neither the content of Rubisco protein nor the activity of Rubisco, as assessed from the A/C_i curve, were significantly different in LL and HL plants. This contrasts with many previous studies of photoacclimation (Björkman, 1981; Bailey *et al.*, 2001).

However, significant differences were observed between the photoacclimation of Orfeo and Arroz. First, the RuBP regeneration capacity was increased in HL only in Orfeo, which is consistent with the elevated capacity of electron transport found in this variety under such conditions. Secondly, a large increase in the number of stomata on the upper adaxial leaf surface was observed only in Orfeo. Thus, it is suggested that, in Orfeo, the increased stomatal conductance arising from the increased stomatal frequency,

Fig. 7. Effect of growth conditions on leaf thickness and palisade development. Black columns, Arroz; white columns, Orfeo. (A) Leaf thickness; (B) palisade depth; (C) palisade cell number. Growth conditions were LL/LT, 300 µmol m^{-2} s^{-1} PAR/22–25 °C. LL/HT, 300 µmol m^{-2} s^{-1} PAR/32–35 °C. HL/LT, 1000 µmol m^{-2} s^{-1} PAR/22–25 °C. HL/HT, 1000 µmol m^{-2} s^{-1} PAR/32–35 °C. Measurements were taken from the mature leaves of at least six individual plants and results represent mean ±standard error (n >24).

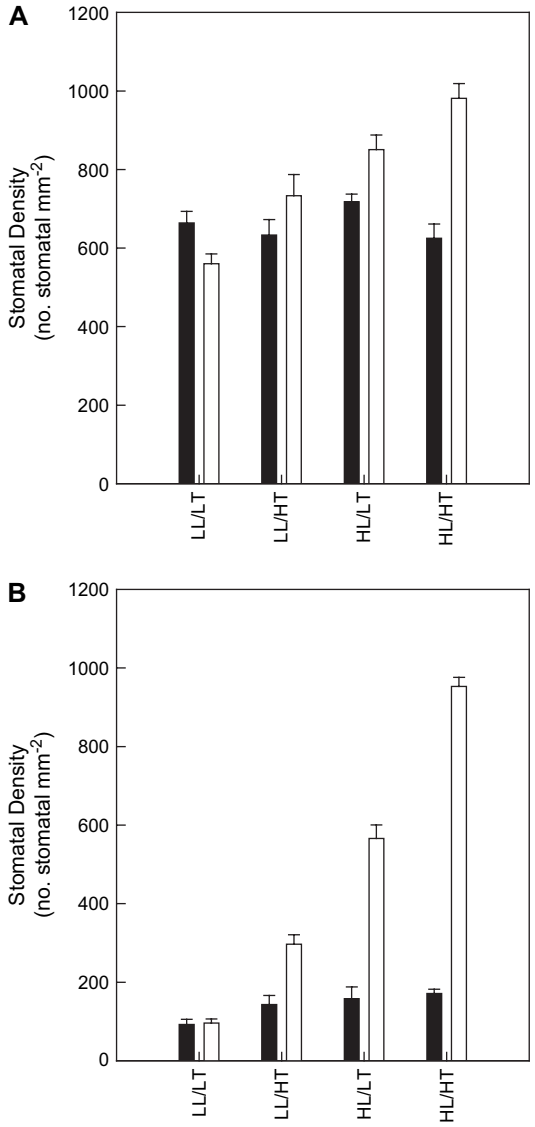


Fig. 8. Effect of growth conditions on stomatal density on the abaxial (A) and adaxial (B) leaf surfaces. Measurements were taken from the mature leaves of at least six individual plants and results represent mean ±standard error (n >24). Arroz, black columns; Orfeo, white columns. For growth conditions, see Fig. 7.

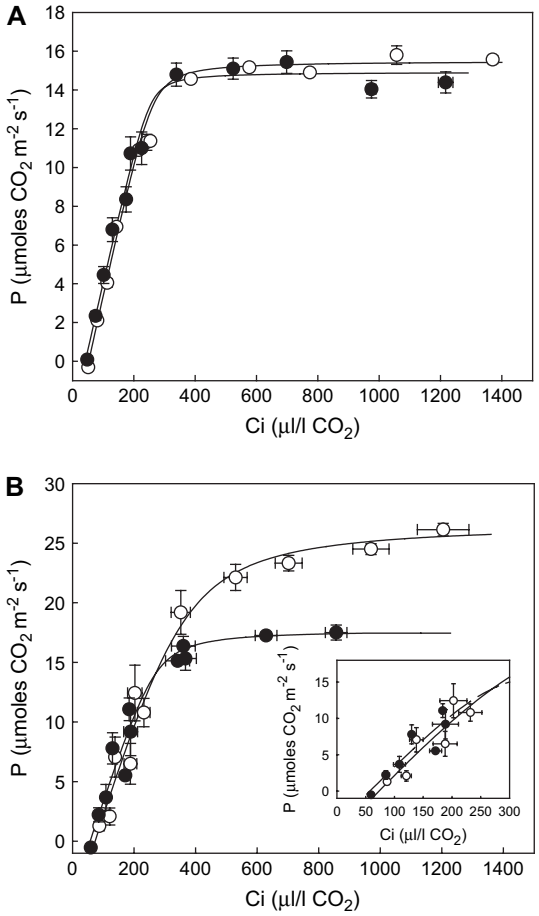


Fig. 9. Light-saturated photosynthetic rate at different calculated internal CO_2 concentrations (C_i) for plants grown under (A) low-light and (B) high-light conditions as described for Fig. 1. The insert in (B) is for a C_i of 0–300 μl l^{-1} CO_2. Open circles, Orfeo; filled circles, Arroz. Measured light intensity was 1500 μmol PAR m^{-2} s^{-1}.

allows an elevated C_i, and that this allows the expression of a higher photosynthetic rate despite an unchanged Rubisco level, utilizing the higher electron transport capacity.

Thus, it is suggested that the key difference between Orfeo and Arroz lies in its stomatal characteristics. A 20-fold increase in the stomatal frequency on the adaxial surface and a 3-fold increase in total stomatal numbers in HL Orfeo compared with HL Arroz, gives a greatly increased potential stomatal conductance. This is borne out by experimental measurements, which indicate a maximum stomatal conductance in HL Orfeo of 0.32 mmol H_2O m^{-2} s^{-1} compared with 0.22 in HL Arroz (see data in Lizano *et al.*, 2006). The importance of the adaxial stomata is perhaps illustrated by the different results obtained when the leaves were illuminated from the underside. Here, at ambient CO_2, the photosynthetic rates of both Orfeo and Arroz were the same, and reduced greatly below the potential capacity observed at saturating CO_2. It is suggested that the adaxial stomata are not effectively opened under these conditions due to a reduced light intensity reaching the guard cells.

However, it is at first sight surprising that Orfeo, the variety with better performance under drought, has the higher stomatal density. In crop plants, high stomatal density is normally related to high values in stomatal conductance (g_s) and high transpiration rates (Davies and Zhang, 1991). However, since stomatal density is defined early during leaf development (Lake *et al.*, 2002), in mature leaves the dynamic control of stomatal opening is more important for control of g_s and water loss. It is known that many species survive water stress by means of effectively retaining water through closure of stomata. As shown in Lizano *et al.* (2006), the stomata of Orfeo appear to be more dynamic. The minimum stomatal conductance under drought was three times lower than that of Arroz, and time for closing

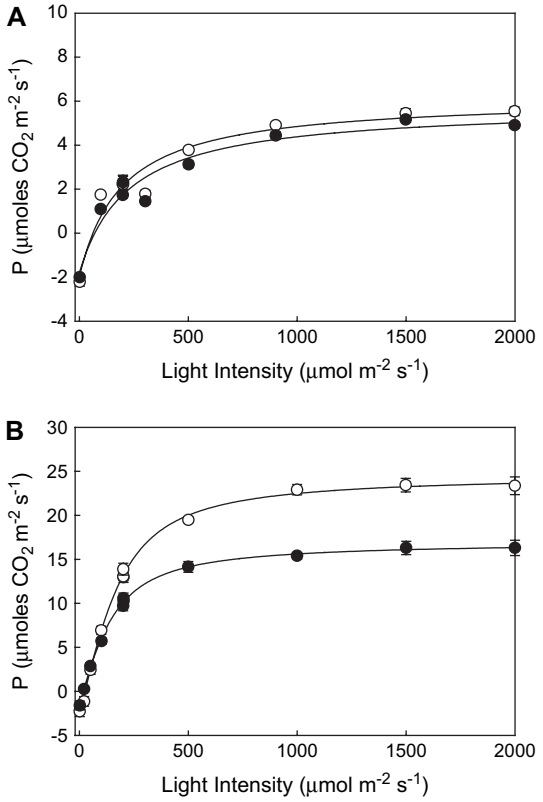


Fig. 10. Photosynthetic rate (*P*) at different light intensities for plants grown under high-light conditions when illuminated from the underside of the leaves: (A) measured at 350 μl l^{-1} CO_2 and (B) measured at 900 μl l^{-1} CO_2. Growth conditions were as in Fig. 1. Open circles, Orfeo. filled circles, Arroz.

was also faster. Thus the Orfeo stomata allow it to have a greater conductance (for enhanced photosynthesis) and a more effective response to drought.

Stomata are generally located on the abaxial leaf surface for light and, therefore, heat avoidance. For beans, however, the fact that high irradiances, high temperature, and also high air to leaf water vapour pressure deficit induces paraheliotropic responses (Donahue, 1990; Yu and Berg, 1994; Pastenes *et al*., 2004, 2005), stomatal location may be less important. Bean leaves move avoiding the incident light, particularly as temperature increases through the day, as an effectively photoprotective response, also maintaining leaf temperature below the ambient (Pastenes *et al*., 2004). Therefore, the strong increase in stomatal frequency on the adaxial surface in the HL/HT Orfeo may not provide a direct target for water loss through incident light, as would be expected from non-moving leaves.

The other strikingly different feature of the leaves of HL-grown Orfeo is the leaf cell composition. In LL the leaves of both varieties are very similar. However, in HL, only the leaves of Arroz show the elongated cells of the palisade layer, which are characteristic of a high light-adapted leaf (Sims and Pearcy, 1992; Oguchi *et al*., 2003; Yano and Terashima, 2004). In Orfeo, the increased leaf thickness is associated with an increase in number of smaller, more rounded cells that resemble instead the spongy mesophyll. In fact, the Orfeo leaf is remarkably symmetrical. At present, the functional significance of the atypical cell composition of HL Orfeo leaves is not understood. It has been shown that the upper cell layers affect light penetration into the leaf (Vogelmann, 1993) and there is significant photoacclimation within the leaf (Terashima and Inoue, 1985; Nishio *et al*., 1993). Therefore, it is possible that the Orfeo leaf structure gives better distribution of photosynthetic activity and light through the leaf. Alternatively, the more symmetrical cell composition may be a result of the even distribution of stomata on the abaxial and adaxial surfaces. For instance, it would be expected that gradients of CO_2 and even photosynthetic products would be diminished in the Orfeo leaf, and these metabolic signals could affect leaf cell development (Yano and Terashima, 2004; Murchie *et al*., 2005). The more even incidence of light on both leaf surfaces, as a result of paraheliotropism, would benefit gas exchange and CO_2 fixation from the even stomatal distribution and a more symmetrical distribution of photosynthetic activity on both leaf surfaces. However, even though both varieties showed leaf movement under drought, only Orfeo shows the atypical stomatal distribution and leaf structure.

These features of stomatal distribution and leaf cell structure, together with an elevated electron transport capacity, are induced in Orfeo under conditions of high light and high temperature when drought stress is also common under field conditions. There are undoubtedly other aspects to the high photoacclimation potential of Orfeo (Lizana *et al*., 2006). The induction of anthocyanin synthesis in HL conditions is one such example. This greater plasticity of Orfeo also includes its sensitivity to ABA and its rapid synthesis of ABA under stress. All of these characteristics are absent in Arroz and, therefore, explain not only why it is more sensitive to drought stress, but also why its productivity is less under conditions of abiotic stress.

It is concluded that the origin of differential stress tolerance may reside in those factors which determine leaf cell development, particularly the stomata. Recently, there has been considerable progress in understanding the signalling pathways that determine stomatal development. The asymmetric cell-division programme governing stomatal development is believed to be controlled by the activity of a MAP kinase cascade, which may itself be regulated by the interaction of a peptide ligand with a receptor-like protein kinase (Nadeau and Sack, 2003; Bergman *et al*., 2004; Gray and Hetherington, 2004). Environmental signals such as light intensity and CO_2 concentration are known to modulate stomatal frequency, but currently little is known about how such environmental signals impact on stomatal development (Gray *et al*., 2000). However, recent work with *Arabidopsis* mutants suggests that the environmental control of stomatal development is regulated by different pathways on the abaxial and adaxial epidermal layers (Lake

et al., 2002). Factors specifying abaxial or adaxial identity of the epidermal layer have been identified (McConnell *et al*., 2001; Fleming, 2005) and may be important in allowing differential control of stomatal development on the abaxial and adaxial surfaces. The genes controlling adaxial stomatal development may be promising targets for the genetic improvement of dicotyledonous plants.

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