Drought effect on photosynthetic activity, osmolyte accumulation and membrane integrity of two *Cicer arietinum* genotypes

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Abstract

Drought was induced in chickpea (*Cicer arietinum* L.) genotypes (ChK 3226 and ILC 3279) differing in yield capacity. Water stress (S1, RWC around 55–50%; S2, RWC ≤ 40%) drastically reduced stomatal conductance (g_s) and net photosynthetic rate (P_N) in both genotypes. ILC 3279 showed greater photosynthetic capacity (A_{max}) decreases. Maximum PSII photochemical efficiency (F_v/F_m), photochemical quenching (q_P), total chlorophylls (Chls) and carotenoids (Cars) content showed stability in both genotypes under stress, but in S2 ILC 3279 presented an increase in basal fluorescence (F_0) and a greater reduction in estimation of quantum yield of linear electron transport (Φ_e) than ChK 3226. Membrane damage evaluated by electrolyte leakage occurred earlier and was greater in ILC 3279. It also presented a decrease of total fatty acids (TFA) along drought, while in ChK 3226 greater amounts of TFA were observed in S1. In rehydration, P_N of S1 plants completely recovered (ILC 3279) or remained slightly below control (ChK 3226). As regards S2 plants, ILC 3279 showed stronger P_N and g_s reductions than ChK 3226, despite both genotypes totally recovered A_{max} and chlorophyll (Chl) *a* fluorescence. ChK 3226 recovered more efficiently from membrane damage. Under control conditions, greater amounts of most of the studied soluble metabolites occurred in ChK 3226 plants. Malate and citrate decreased with water stress (S2) in both genotypes. Sucrose and pinitol (that had a higher concentration than sucrose in both genotypes) increased in ILC 3279 (S1 and S2), and decreased in ChK 3226 (S2). In ILC 3279 proline and asparagine followed similar patterns. Genotypes showed a similar shoot dry mass (DM) in control plants, but root DM was higher in ChK 3226. Drought reduced root and shoot DM in ChK 3226 already under S1, while in ILC 3279 root DM was unaffected by drought and shoot biomass decreased only in S2. Root/shoot ratio was always higher in ChK 3226 but tended to decrease under stress, while the opposite was observed in ILC 3279. No pods were obtained from control plants of both genotypes, or droughted ILC 3279 plants. ChK 3226 produced pods under S1 (higher yield) and S2. Under stress conditions, ChK 3226 was less affected in photosynthetic activity and membrane integrity, showing a better tolerance to drought. This agrees with the better yield of this genotype under water stress. Distinct strategies seem to underlie the different physiological responses of the two genotypes to water deficit. In spite of its significant solutes accumulation, ILC 3279 was more affected in photosynthetic activity and membrane integrity during water stress than ChK 3226, which showed better yield under drought. A relation could not be established between solutes accumulation of ILC 3279 and yield.

Additional key words: chlorophyll fluorescence, electrolyte leakage, membrane lipids, water stress.

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Abbreviations: A_{max} – photosynthetic capacity; Asn – asparagine; C – control; Cars – carotenoids; Ci – citrate; Chl(s) – chlorophyll(s); C18:3 – linolenic acid; DM – dry mass; FM – fresh mass; TM – turgid mass; F_0 – basal fluorescence; F_v/F_m – maximum PSII photochemical efficiency; g_s – stomatal conductance rate; Ma – malate; Pi – pinitol; P_N – net photosynthetic rate; Pro – proline; q_P – photochemical quenching; Rec – recovery; R_D – dark respiration rate; RWC – relative water content; Suc – sucrose; S1, S2 – water stress levels 1 and 2; TFA – total fatty acids; Φ_e – estimation of quantum yield of linear electron transport.+
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Introduction

Like many Mediterranean legume crops, chickpea (*Cicer arietinum* L.) is usually subjected to drought stress at the end of the growing season (Gaur *et al*. 2008). The influence of reduced water availability on crop productivity depends on the genotype tolerance to this constraint. Soil water deficit, high leaf-to-air water vapour pressure deficit (Schulze 1986) and leaf water deficit (Hsiao 1973) have been shown to induce stomatal closure, which is the primary cause of photosynthetic reduction (Chaves 1991). Nonstomatal effects on photosynthetic and respiratory processes can occur at relative leaf water content below 50–70% (Kaiser 1987). Nevertheless, some plants can develop strategies or responses to maintain assimilatory activities under such conditions.

Some of these traits may contribute to maintain high leaf water potential, which can minimize the negative effects of water deficit on photosynthetic metabolism and plant yield. Osmotic adjustment has been considered a drought-adaptive mechanism in crop plants, contributing to yield stability of chickpea in dry environments (Morgan *et al*. 1991, Moinuddin and Khanna-Chopra 2004). Several osmolytes may be involved in osmotic adjustment, namely sugars, amino acids and organic acids (Morgan 1992). Among these solutes, proline may influence protein solvation under dehydration (Paleg *et al*. 1984), functioning as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989) and stabilizing membranes by interacting with phospholipids (Rudolph *et al*. 1986). Membrane stabilization may also be achieved through preservation of membrane lipids or changes in lipid composition (Pham Thi *et al*. 1990). These changes may contribute to maintain membrane integrity and stability, which are key factors to ensure cell compartmentation and metabolism under adverse conditions (Leshem 1992). Membrane damage in several legumes under water stress has been assessed through electrolyte leakage test (Pham Thi *et al*. 1990, Matos *et al*. 2002).

In this work two chickpea genotypes differing in yield capacity under drought (Maçãs 2003) were compared to determine the effects of water stress on photosynthetic activity, osmolyte accumulation, membrane integrity, and biomass production. Membrane integrity was evaluated through electrolyte leakage and total fatty acids (TFA) content and composition, to assess the protoplasmic tolerance of these two genotypes. Changes in osmotic solutes, which may be related to the maintenance of membrane stability and, hence, photosynthetic activity, were also evaluated. The information obtained will enable characterisation of these genotypes under water shortage and will contribute to breeding programs aiming at drought adaptation.

Materials and methods

Plant material and growth conditions: Two genotypes of *C. arietinum* L., ChK 3226 from Afghanistan and ILC 3279 from Russia, were used. Three seeds were sown in 1-l pots filled with a mixture of peat (Triohum tray substrate): vermiculite (3:1). After germination 2 plantlets were left in each pot and maintained in a greenhouse under natural light conditions, mean daily temperatures of 30ºC and relative humidity values between 60% (early morning) and 40% (midday). One-month-old plants were irrigated with a modified Hoagland and Snyder (1933) solution (two-fold micronutrients) and transferred to growth chambers (*700 EDTU*, *ARALAB*, Portugal) under controlled conditions of light [650 μmol(quantum) m^{-2} s^{-1}], temperature (25/20ºC, day/night), air humidity (70%) and photoperiod (12 h).

Water stress treatments: Adult plants (1.5 months old, *ca*. 50 cm high) were split in 3 groups: in the 1st group (control) plants were irrigated twice a week to maintain high relative water content (RWC); in the 2nd (S1) and 3rd (S2) groups, plants were subjected to water shortage by withholding irrigation for several days. A correlation between field capacity and RWC values was previously established. Pots were weighed daily until soil water content corresponded to low leaf RWC values (*ca*. 55–50% for S1 and ≤ 40% for S2). Rewatering was achieved by full irrigating S1 and S2 plants. Measurements were carried out in control, S1, S2, and rewatered plants. In rehydration (Rec), gas exchange (P_N and g_s) was measured 1 and 3 days (Rec-1 and Rec-3) after rewatering of S1 and S2 plants. A_{max} and fluorescence parameters were obtained after 3 days of rehydration only in S2 plants. Leaf samples for lipid analysis, electrolyte leakage, and soluble metabolites were collected 6 days (Rec-6) after rewatering of S2 plants.

RWC was calculated on four replicates as RWC = [(FM–DM)/(TM–DM)] × 100, where FM is the fresh mass of 2 leaves of each pot and TM is the turgid mass after overnight rehydration of the leaves, kept in darkness in dishes with distilled water at 4ºC to minimise respiration losses, until they reached a constant mass. DM was obtained 48 h after keeping the turgid leaves at 80ºC in an oven.

Gas exchanges and fluorescence measurements: P_N, dark respiration (R_D), and g_s were measured in the first fully expanded leaf, using a portable CO_2/H_2O gas-exchange system *LI-6200* (*LI-COR*, Lincoln, U.S.A.) in a 0.25-l chamber. P_N and g_s were measured under 720 μmol(quantum) m^{-2} s^{-1}, 26ºC air temperature, 45% relative humidity and *ca.* 350 ppm CO_2. R_D measurements

were performed on control and S1 plants, which were left for stabilization under dark conditions for at least 15 min after gas-exchange analysis to avoid any light-enhanced dark respiration. A_{max} was determined in leaflets using a Clark-type leaf disc oxygen electrode (*LD2/2 Hansatech*, Kings Lynn, U.K.), at 25ºC, under light [800 µmol (quantum) m^{-2} s^{-1}] and CO_2 (*ca*. 7% supplied by 400 µl of 2M $KHCO_3$) saturating conditions. Irradiance was provided by a Björkman halogen lamp (*Hansatech, Ltd*.). Chl *a* fluorescence parameters were measured on leaflets placed inside a *LD2/2* system, using a *PAM 2000* system (*Walz*, Effeltrich, Germany), under saturating CO_2 conditions (*ca*. 7% supplied by 400 µl of 2M $KHCO_3$,) and 25ºC, as previously described (Lidon *et al*. 1993, Ramalho *et al*. 2003). Briefly, measurements of F_0 from the antennae and F_v/F_m were performed in overnight-dark-adapted leaflets from the same leaf used for A_{max}. F_0 denotes the fluorescence emission by the excited Chl *a* molecules before the excitions have migrated to the reaction centres and was determined using a weak light measuring beam [*ca*. 0.1 µmol(quantum) m^{-2} s^{-1}]. F_v/F_m represents the photochemical efficiency of PSII and was obtained using a saturating flash (0.8 s) of 9,500 µmol(quantum) m^{-2} s^{-1}. F_0' needed for q_P determination was obtained imediately after the switch off of the actinic light, before the first fast phase of fluorescence relaxation kinetics in the dark. The estimation of the quantum yield of linear electron transport (Φ_e) and the photochemical quenching coefficient (q_P) were obtained under photo-synthetic steady-state conditions of 570 µmol(quantum) m^{-2} s^{-1}, superimposing saturating flashes of *ca*. 9,500 µmol(quantum) m^{-2} s^{-1} and a duration of 0.8 s. The Φ_e is a good measure of the quantum efficiency of noncyclic electron transport and was obtained according to Genty *et al*. (1989), while q_P denoting the proportion of energy trapped by PSII open centres and driven to photochemical events, was calculated according to Van Kooten and Snell (1990).

Pigment analysis: Immediately after A_{max} and Chl fluorescence measurements, leaflets (*ca*. 1.5 cm^2) were used for pigment determination. A previous correlation between leaf fresh mass and leaf area was established using an area meter (*MK2, Delta T Device*, U.K.). Chls and Cars were extracted in 80% acetone at 4ºC. After centrifugation (10,000 × *g*, 10 min, 4ºC), the pigment content of the supernatant was estimated spectrophoto-metrically according to Lichtenthaler (1987).

Electrolyte leakage measurements: Sixteen leaflets approximately from the same size (*ca*. 0.5 cm^2 each) were detached from fully expanded leaves, rinsed with water and floated on deionised water. Conductivity was measured after 22 h of floating at room temperature, with a conductimeter *Crison 522* (*Crison Instruments*, *S.A*., Spain). Total conductivity was measured after keeping the samples in an oven (90ºC) for 2 h. Membrane leakage for each sample was expressed as a percentage of the conductivity after 22 h of floating in relation to its total conductivity.

Lipid analysis: Frozen leaf tissue samples (3–4 g FM) were boiled for 2 min in distilled water, in order to stop lipolytic activities. Lipids were extracted with a mixture of chloroform/methanol/water (1:1:1, v:v:v) according to Allen *et al*. (1966). Fatty acids were saponified and methylated with BF_3 (*Merck*) using heptadecanoic acid (C17:0) as an internal standard and analysed using a gas chromatograph (*Unicam 610 Series, Unicam Ltd*., U.K.), equipped with a flame-ionization detector. Separation was performed using a *DB-Wax* (*J&W Scientific*, U.S.A) capillary column (0.25 mm × 30 m, 0.25 µm), as described in Matos *et al*. (2002). Temperature was programmed to raise from 80 to 200ºC at 12ºC min^{-1}, after 2 min at the initial temperature. Injector and detector temperatures were 200 and 250ºC, respectively. Carrier gas was hydrogen (1 ml min^{-1}, split ratio of 1:100 of the sample). Fatty acids were identified by comparison with known *Sigma* standards.

Soluble metabolite analysis: Frozen leaf samples (0.5–2.5 g, from 7 to 30 leaflets) were ground to a fine powder on liquid nitrogen in a mortar and dropped into boiling water for 5 min. The slush was centrifuged, the recovered supernatant lyophilized and the residue resuspended in 4 ml of an aqueous solution containing D_2O (5.8 M), Na_2EDTA (2.5 mM) and NaN_3 (2.5 mM). The qualitative and quantitative characterization of extracted osmotic solutes was carried out by ^{13}C-nuclear magnetic resonance (^{13}C-NMR), using dioxan as an internal concentration standard (Passarinho *et al*. 2006). Spectra were obtained at 75.47 MHz on a *Bruker AMX300* spectrometer (Ettlingen, Germany) using a 10 mm diameter broadband probe head.

Biomass quantification: After the end of the water-shortage period, plants (C, S1, and S2) were maintained under control irrigation until they were harvested. DM of roots, shoots, and pods was determined after oven drying for 48 h at 70ºC.

Statistical analysis: Results were statistically analyzed by the *Statistix* program *version 7* (*Analytical Software*, USA, 1998).

Results

Gas exchange measurements: Water stress (S1 and S2), whose values were referred in Table 1, strongly reduced g_s (*ca*. 88%) in both genotypes, resulting in negative P_N values (Fig. 1). After the 1st and 3rd days of rehydration (Fig. 1), P_N of S1 plants completely recovered (ILC 3279) or presented values slightly below control (ChK 3226). Gradual g_s increases were observed from the 1st to the 3rd day in rewatered S1 plants of both genotypes. In rehydrated S2 plants of ILC 3279, P_N and g_s showed worse recoveries as compared to ChK 3226. R_D suffered a reduction (12%) in ChK 3226, decreasing from 5.5 ± 0.4 µmol(O_2) m^{-2} s^{-1} in the control to 4.8 ± 0.8 µmol(O_2) m^{-2} s^{-1} in S1 conditions. Contrarily, under the same conditions, in ILC 3279 it increased (91%) from 3.4 ± 0.3 µmol(O_2) m^{-2} s^{-1} to 6.5 ± 0.6 µmol(O_2) m^{-2} s^{-1}. A_{max} was severely reduced in ILC 3279 (55% and 65% for S1 and S2, respectively) when compared to ChK 3226 (29% and 43% for S1 and S2, respectively). Both genotypes showed a complete A_{max} recovery in the 3rd day of rehydration (Fig. 1).

Fluorescence measurements: As regards F_0, ILC 3279 presented an increase of 19.4% under S2 (Fig. 2). Similar tendencies and values were observed in both genotypes for F_v/F_m and q_P which showed a high stability to water stress. The estimation of the quantum yield of linear electron transport (Φ_e) decreased *ca.* 19% in ChK 3226 and 26% in ILC 3279 under S2 conditions (Fig. 2). Recovery of all these parameters was complete after rehydration.

Pigments analysis: Total Chls (Table 1) did not decrease under water stress (S1 and S2) in both genotypes, but showed nonsignificant rises of 19% (S1) and 7% (S2) in ILC 3279. Under rehydration the ILC 3279 maintained values higher (13%) than in control, but a significant decrease of 22% was found in ChK 3226 plants. The ratio Chl *a*/*b* remained unaltered under water stress (S1 and S2) and after rehydration in both genotypes. Total Cars did not differ between treatments, but under S1 they tended to decrease in ChK 3236, contrarily to what was observed in ILC 3279. The Chl (*a*+*b*)/Cars ratio was not significantly affected, denoting the absence of a

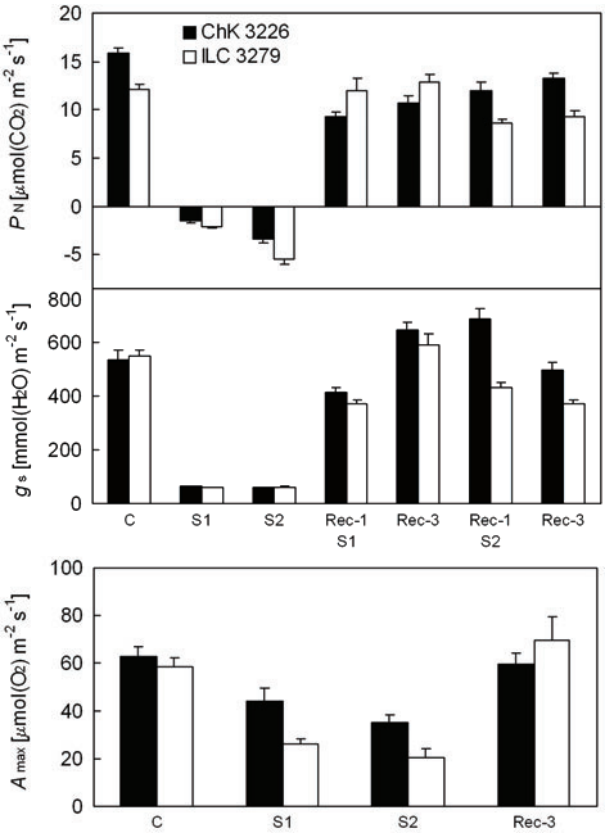

Fig. 1. Changes in net photosynthetic rate (P_N), stomatal conductance (g_s) and photosynthetic capacity (A_{max}) of two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226) under well watering (C), water stress (S1, RWC 55–50%; S2, RWC ≤ 40%) and recovery Rec-1 and Rec-3 (one and three days after rewatering for S1 and S2 plants); A_{max} recovery was measured in the 3rd day in S2 plants. Bars represent the mean ± SE (n = 9).

Table 1. Changes in relative water content (RWC) and photosynthetic pigments of two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226) under well watering (C), water stress (S1, RWC 55–50%; S2, RWC ≤40%) and recovery (Rec-3, three days after rewatering S2 plants). Values are means (n = 3). Different letters express significant different results between dehydration levels in the same genotype (a, b) or between genotypes with the same dehydration level (r, s). Cars – carotenoids; Chl – chlorophyll.

		RWC [%]	Chl (*a*+*b*) [mg m^{-2}]	Chl (*a*/*b*)	Cars [mg m^{-2}]	Chl (*a*+*b*)/Cars
ChK 3236	C	84.8^{ar}	609^{ar}	3.9^{abr}	122^{ar}	5.1^{ar}
	S1	53.5^{br}	605^{a}	3.7^{bs}	110^{ar}	5.5^{ar}
	S2	38.0^{cr}	626^{ar}	3.7^{br}	114^{ar}	5.5^{ar}
	Rec-3	78.3^{ar}	472^{br}	4.0^{ar}	96^{ar}	4.9^{ar}
ILC 3279	C	85.5^{ar}	498^{ar}	3.7^{ar}	91^{ar}	5.6^{ar}
	S1	48.8^{br}	595^{ar}	3.8^{ar}	109^{ar}	5.4^{ar}
	S2	40.0^{cr}	533^{ar}	3.6^{ar}	100^{ar}	5.3^{abr}
	Rec-3	80.7^{ar}	561^{ar}	3.9^{ar}	116^{ar}	4.8^{br}

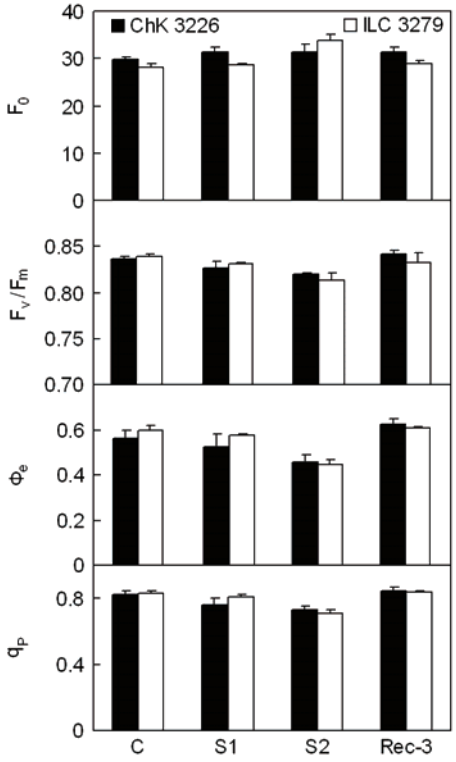

Fig. 2. Changes in basal fluorescence (F_0), photochemical efficiency of PSII (F_v/F_m), quantum yield of PSII electron transport (Φ_e), and photochemical quenching (q_P) of two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226) under well watering (C), water stress (S1, RWC 55-50%; S2, RWC ≤40%) and recovery (Rec-3, three days after rewatering S2 plants). Bars represent the mean ± SE (n = 3–5).

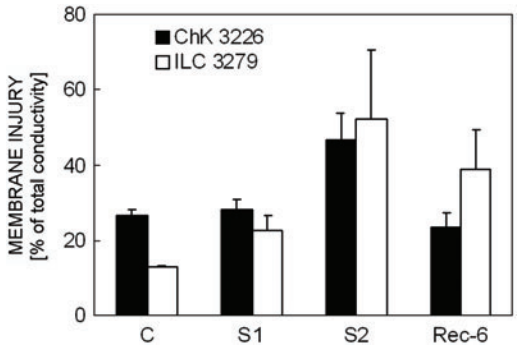

Fig. 3. Evaluation of membrane injury in two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226) through electrolyte leakage of leaf discs in deionised water, expressed as percentage of total conductivity. Discs were collected from well watered (C), water-stressed (S1, RWC 55–50%; S2 RWC ≤ 40%) and rehydrated plants (Rec-6, six days after rewatering S2 plants). Bars represent the mean ± SE (n = 4).

significant reinforcement of dissipation over capture energy pigments. Only under recovery ILC 3279 presented a significant 14% decrease of that ratio, as compared to its control, mostly due to the 27% rise in total Cars observed in this period.

Electrolyte leakage measurement: Water shortage increased electrolyte leakage in ILC 3279 both under S1 (75%) and S2 (300%) conditions (Fig. 3). After rehydration, conductivity values in this genotype were still significantly higher (198%) than in control. ChK 3226 was affected only under S2 (75% increase) and showed a complete recovery after rewatering.

Lipid analysis: ILC 3279 presented a gradual decrease of total fatty acids (TFA) with increasing drought severity (7.5% for S1 and 19% for S2), while in ChK 3226, TFA content increased 14% under S1, and decreased 14% under S2 (Fig. 4). After rehydration, this genotype presented a total recovery (values similar to control) unlike ILC 3279. As regards changes in major fatty acids (Table 2), palmitic acid (C16:0) remained unaltered in ChK 3226 droughted plants, but it increased in ILC 3279 under S1. Linoleic acid (C18:2) decreased in both

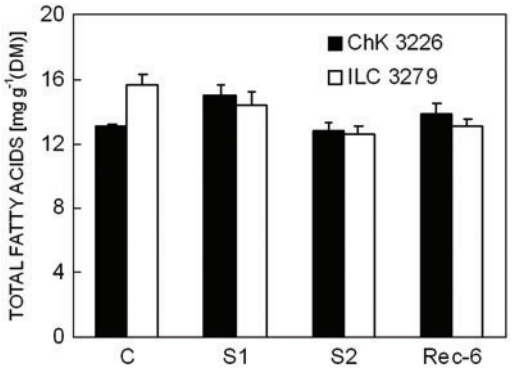

Fig. 4. Changes in the total fatty acids content (TFA) in leaves of two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226) under well watering (C), water stress (S1, RWC 55–50%; S2 RWC ≤ 40%) and recovery (Rec-6, six days after rewatering S2 plants). Bars represent the mean ± SE (n = 4).

Table 2. Changes in major fatty acids [%] of total lipids of leaves of two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226) under well watered (C), water stress (S1, RWC 55–50%; S2 RWC ≤ 40%) and recovery (Rec-6, six days after rewatering S2 plants). C16:0, palmitic acid; C18:2, linoleic acid; C18:3, linolenic acid. Values are means (n = 4). Different letters express significant different results between dehydration levels in the same genotype (a, b) or between genotypes with the same dehydration level (r, s).

		C16:0 [%]	C18:2 [%]	C18:3 [%]
ChK 3226	C	13.6^{ar}	6.4^{as}	72.9^{abr}
	S1	14.6^{ar}	4.8^{br}	75.3^{ar}
	S2	13.9^{ar}	4.2^{br}	74.8^{ar}
	Rec-6	14.1^{ar}	7.5^{ar}	70.3^{bs}
ILC 3279	C	13.3^{br}	7.9^{ar}	72.7^{br}
	S1	14.9^{ar}	5.3^{br}	75.7^{ar}
	S2	14.0^{abr}	4.9^{br}	74.8^{abr}
	Rec-6	13.9^{abr}	7.2^{ar}	72.5^{br}

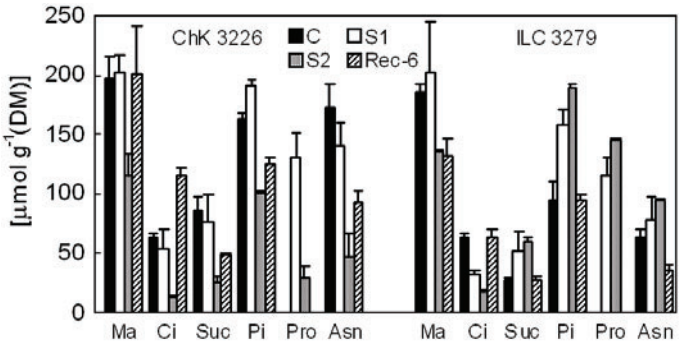

Fig. 5. Changes in the content of soluble metabolites in leaves of two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226) under well watering (C), water stress (S1, RWC 55–50%; S2 RWC ≤ 40%) and recovery (Rec-6, six days after rewatering S2 plants). Ma – malic acid; Ci – citric acid; Suc – sucrose; Pi – pinitol; Pro – proline; Asn – asparagine. Bars represent the mean values ± SE of two distinct leaf extracts.

Table 3. Effects of water stress (S1, RWC 55–50%; S2, RWC ≤ 40%) on biomass parameters, expressed as dry mass (DM) per pot, in two *Cicer arietinum* genotypes (ILC 3279 and ChK 3226). Control (C) corresponds to well hydrated plants. Values are means (n = 3). Different letters express significant different results between dehydration levels in the same genotype (a, b) or between genotypes with the same dehydration level (r, s).

		Shoot DM [g]	Root DM [g]	Root/Shoot	Pods DM [g]	Number of pods
ChK 3236	C	22.5^{ar}	21.5^{ar}	0.95^{ar}	0.0^{br}	0.0^{cr}
	S1	15.8^{br}	12.4^{br}	0.78^{ar}	2.6^{ar}	39.3^{ar}
	S2	13.5^{br}	11.2^{br}	0.84^{ar}	2.1^{abr}	22.0^{br}
ILC 3279	C	22.1^{ar}	12.2^{as}	0.55^{bs}	0.0^{ar}	0.0^{ar}
	S1	21.4^{ar}	12.1^{ar}	0.57^{abs}	0.0^{as}	0.0^{as}
	S2	15.0^{br}	11.4^{ar}	0.79^{ar}	0.0^{as}	0.0^{as}

genotypes under S1 and S2, returning to initial values after re-watering. Linolenic acid (C18:3) increased in ILC 3279 under S1. A similar tendency was observed in ChK 3226. During rehydration both genotypes reduced the percentage of C18:3 in relation to S1 values.

Soluble metabolites analysis: As regards the amount of the soluble metabolites in the leaves, well watered plants of ILC 3279 had a lower value [435 µmol g^{-1}(DM)] than ChK 3226 plants [685 µmol g^{-1}(DM)]. An increase of 48% was observed under S1 and S2, in ILC 3279. ChK 3226 showed an increase under S1 (16%) followed by a significant reduction (58%) under S2. Malate, the most abundant osmolyte in both genotypes (Fig. 5), decreased by 40% (ChK 3226) and 27% (ILC 3279) only under S2. After rehydration, ChK 3226 totally recovered, but ILC 3279 maintained the lowered S2 values.

Citrate decreased in both genotypes along dehydration (*ca*. 70–80%). Recovery was complete, particularly in the case of ChK 3226 (twice the value of the control).

Sucrose increased with water stress in ILC 3279 reaching a maximum rise of 112% in S2, while it decreased in ChK 3226 (70% for S2). After rehydration, ILC 3279 recovered to control values, while ChK 3226 remained at only 56% of the initial value.

Control plants of ChK 3226 had greater amounts of pinitol than ILC 3279 (Fig. 5). Under S1, an increase occurred (67% and 17% for ILC 3279 and ChK 3226, respectively). A further increase was observed in ILC 3279 under S2, while ChK 3226 showed a decrease. In this genotype, pinitol content increased during recovery, contrarily to ILC 3279 where a decrease was observed leading to values similar to control. Proline was not detected in well watered and rehydrated plants (Fig. 5). Under S1 it significantly increased in both genotypes. Further dehydration of leaves (S2) resulted in an increase in ILC 3279 (24%) and a decrease in ChK 3226 (77%). Asparagine content gradually increased in ILC 3279 with water deficit (50% higher under S2), while it decreased in ChK 3226 under S1 and S2 (73%). After rewatering, this amino acid partially recovered (50% of control) in ChK 3226 and dropped to values below control in ILC 3279.

Biomass production: Both genotypes showed similar shoot DM under control conditions, but were differently affected by water shortage. A progressive reduction (30% for S1 and 40% for S2) occurred in ChK 3226, while in ILC 3279 a reduction (68%) was observed only under S2 (Table 3). Root DM was 43% higher in ChK 3226 than in ILC 3279 in control plants. However ChK 3226 presented dramatic reductions of 42% and 48% under S1 and S2, respectively, while in ILC 3279 root DM remained stable under drought. The ratio root/shoot was always higher in ChK 3226. Under drought this ratio increased in ILC 3279 (S2) and tended to decrease in ChK 3226. By the end of the experiment, no pods were obtained from well irrigated plants of both genotypes, or droughted ILC 3279 plants. However, ChK 3226 plants produced pods under S1 and S2, presenting a higher pod number and pod DM under S1.

Discussion

Severe dehydration is likely to induce changes at the photosynthetic machinery level that hamper photosynthetic processes (Kaiser 1987). Despite chickpea being considered one of the most drought-tolerant of the cool season food legumes (Singh 1993), under low leaf RWC values (S1 and S2 stress conditions) both chickpea genotypes showed g_s reductions which strongly affected CO_2 assimilation, as suggested by the negative P_N rates. R_D response to water stress (S1) differed in the two genotypes, increasing in ILC 3279 and slightly reducing in ChK 3226. Several authors have reported increases in R_D rates due to water deficit (Iljin 1957, Boyer 1965, Galmés *et al*. 2007a). The effect of drought on R_D is not clear and several authors have reported different responses that can be either increases, maintenance or decreases (Vieira da Silva 1976, Galmés *et al.* 2007b). However, the ability to maintain respiration metabolism as a source of energy to the cell, when photosynthesis is low, can represent a positive trait for plants survival under stress, as suggested for cold-tolerant plants (Larcher 1981). As regards A_{max}, ILC 3279 was more affected than ChK 3226 along the stress treatments, but both genotypes fully recovered after rehydration. This can be partially explained by the absence of irreversible damage of PSII centres thus reflecting a high resistance to water stress, as reported for other legumes (Cornic *et al*. 1987). In fact, some rise in F_0 values (only in ILC 3279) was observed, probably indicating a tendency of higher excitation losses during the transfer from the antennae to the reaction centre (Lidon *et al.* 1993), but no concomitant significant reductions were observed on F_v/F_m, showing the absence of photoinhibitory impairments (Ramalho *et al*. 2000). Nevertheless, some impairment in the thylakoid electron transport, as estimated through Φ_e, was observed in both genotypes under S2 conditions, stronger in ILC 3279, thus contributing to the observed impact on A_{max} (Fig. 1).

ChK 3226 showed a higher protoplasmic resistance to dehydration as inferred from a lower impact on membranes, evaluated by membrane leakage and lipid content. Under S1 an enhanced lipid synthesis occurred, which has been considered as a mechanism to improve plants adaptation to adverse environmental conditions, such as water stress (Harwood 1997). It may allow the plant to cope with subsequent severe dehydration and support a better recovery after rehydration, as observed in ChK 3226. Water stress may induce transient conformational modifications of membrane structure at biophysical level, which may be reversible as soon as external conditions become more favourable (Leshem 1992). Changes in membrane permeability may reflect lipid degradation through enzymatic and/or oxidative mechanisms (Sahsah *et al*. 1998). In this case an efficient lipid turnover and/or lipid repair mechanisms would contribute to plants survival.

Qualitative lipid traits, as is the case of a higher C18:3 percentage in ILC 3279 under S1, may also result from lipid turnover (Harwood 1997) and play a role in plant adaptation to adverse conditions. The concomitant lowering of linoleic acid (C18:2) observed in both genotypes under drought also denotes an enhanced biosynthesis of C18:3 (Harwood 1997). This occurs by the action of increased Δ15-desaturase on linoleic acid (C18:2) to give C18:3. This unsaturated fatty acid, highly abundant in photosynthetic membranes, contains double bonds essential to membrane fluidity and maintenance of cellular activity under water shortage (Leshem 1992). Unsaturation level of polar lipids has been reported to remain unchanged or increase in drought-resistant cultivars of cowpea (Monteiro de Paula *et al*. 1990) and *Arabidopsis* (Gigon *et al*. 2004). However, double bonds of polyunsaturated fatty acids are also preferential targets for reactive oxygen species or enzymatic oxidative chain reactions under stress (Sahsah *et al*. 1998), which lead to cell decompartmentation and death. This could explain the higher membrane damage observed in ILC 3279. Nevertheless, this genotype also presents stable pigments contents and good vegetative performance, suggesting the presence of an efficient antioxidative mechanism that allowed a good recovery. Effectively a decrease in Chl (*a*+*b*)/Cars ratio was found in ILC 3279 after rehydration, although the content of Cars in both genotypes is not significantly different in all treatments. This reduction was due to a preferential increase of Cars relatively to total Chls. Furthermore, it seems noteworthy that in ILC 3279 both capture and dissipation pigments presented a tendency to increase during drought and upon rehydration conditions, suggesting an adaptation response to reduce impact in photosynthetic structures.

Chl stability was observed in both genotypes under drought and may be used as an indicator of drought tolerance (Arunyanark *et al*. 2008). Leaf Chl content is often highly correlated with leaf nitrogen content, photosynthetic capacity and ribulose biphosphate carboxylase activity (Bauerle *et al*. 2004). Photosynthetic pigments of the apical leaves, in which the present results were obtained, may have been protected by the remobilization of nutrients from the other parts of the plant, such as lower leaves, which became senescent and showed a strong abscission rate (data not shown). This could be a reason why total Chls and Cars did not decrease under water stress (S1 and S2).

Malate and citrate did not seem to be involved in *C. arietinum* osmotic adjustment as their concentration was maintained under S1 and decreased under severe water stress in both genotypes. Similar results were found in *Lupinus albus* subjected to drought (Pinheiro *et al*. 2004). Decreases of malate may be related with reductions observed in photosynthetic capacity. This organic acid is an end product of photosynthesis and it

plays an important role in plant cell physiology (Patonnier *et al*. 1999), being considered a good indicator of the photosynthetic capacity (Lance and Rustin 1984). After rehydration ChK 3226 completely recovered malate and A_{max}, whereas ILC 3279 maintained a decreased malate content despite the complete recovery of A_{max}.

As regards sucrose content, a depletion of soluble sugars in leaves, as observed in ChK 3226, was previously reported, suggesting that water deficit had a higher effect on CO_2 assimilation than on translocation and use (Hanson and Hitz 1982, Huber *et al*. 1984). Other authors reported that soluble sugars accumulation in leaves during water stress may contribute to osmoregulation (Jones *et al*. 1980, Munns and Weir 1981), what could be the case in ILC 3279. This sugar accumulation may result from the conversion of starch to sucrose, which is stimulated by short-term water stress (Fox and Geiger 1985), the increased activation of sucrose-phosphate synthase and a decrease on sucrose export rate (Quick *et al*. 1992).

Pinitol presented higher concentrations than sucrose, and increased under S1 in both genotypes. Several authors have suggested its involvement in drought tolerance (Keller and Ludlow 1993, McManus *et al*. 2000), high-temperature tolerance (Guo and Oosterhuis 1995) and/or as a transport and storage form of photosynthetically derived sugar. McManus *et al*. (2000) found that pinitol was the major soluble carbohydrate present in water-sufficient leaf tissue of white clover and after a long period of water stress. In soybean plants, high-temperature stress significantly increased plant pinitol content (Guo and Oosterhuis 1995). Our results suggest pinitol may function as an osmotic solute in *C. arietinum*, particularly in ILC 3279 where a gradual increase was observed with drought severity, and returning to lower values (similar to control) after a rehydration period. The decrease observed in ChK 3226 under S2 may correspond to a transient metabolisation process which is partly inverted under recovery.

Proline is considered a major metabolite in osmoregulation of many plant species (Rhodes 1987), including legumes, which seems to be the case of ILC 3279. Effectively its concentration strongly raised as water deficit increased, becoming the major osmolyte after pinitol under S2. Accumulated proline may act as a cytosolute, an osmoprotectant, and a protective agent for cytosolic enzymes and various cellular structures (Larher *et al*. 1993), although its precise role is still not clear, especially in higher plants (Malencic *et al*. 2003). In Chk 3226, a different pattern was observed, with an increase under S1 followed by a reduction in S2. This reduction probably corresponds to a metabolisation of proline to form proline oxidation products, as proposed by Stewart and Boggess (1978) for barley leaves. The absence of proline after recovery suggests an activation of this amino acid catabolism in both genotypes under rehydration conditions. Proline synthetized during water deficit has also been ascribed the role of an organic nitrogen reserve to be used during recovery (Malencic *et al*. 2003).

Water stress reduced asparagine in ChK 3226 plants while it increased in ILC 3279. Several roles have been suggested to explain changes in asparagine content. In temperate legumes, asparagine is the main molecule used to transport reduced nitrogen within the plant (Diaz *et al*. 2005). The presence of asparagine in excess is considered to be a good marker of protein degradation under stress (Genix *et al*. 1990). An increase in asparagine content in *Brassica napus* has been reported in response to osmotic stress (Good and Zaplachinski 1994). It also increased in the stem of *L. albus* plants submitted to water stress, but not in young leaves (Pinheiro *et al*. 2004).

The high reductions in A_{max}, g_s and P_N due to water stress differently affected biomass production of the two genotypes. Whereas in ChK 3226 shoot and root DM were already significantly reduced under S1 conditions (this reduction was maintained under S2), in ILC 3279 effects were only observed in shoot under S2, increasing root/shoot ratio. However this genotype was unable to produce pods, unlike ChK 3226. The better root system in well irrigated ChK 3226 plants may confer a higher capacity to cope with water shortage. The higher content of osmolytes found in ChK 3226 under well irrigated conditions could confer a good capacity to deal with drought. However, under these conditions, in a general way, ChK 3226 does not increase osmolytes but rather uses/metabolizes them, recovering after rehydration. Contrarily, ILC 3279 responded to stress imposition (S1, S2) increasing the content of some soluble metabolites (mainly sucrose, pinitol and proline) that return to values close to control after irrigation. This suggests different responses to drought.

Genotypic variation in expression of osmotic adjustment under water deficit has also been observed for several crops (Jongdee *et al*. 2002, Silva *et al*. 2006). A positive correlation between osmotic adjustment capacity and yield was reported in wheat genotypes cultivated under field conditions (Silva *et al*. 2006). However, recent research has been contradictory relatively to the role of this process in preventing yield reductions in chickpea cultivars exposed to terminal drought (Turner *et al*. 2007). As concerns our results, osmolytes accumulation under limited water availability is probably less related with better yield than the amount of solutes in well watered plants.

Conclusion: Distinct strategies seem to underlie the different physiological responses of the two genotypes to water deficit. Both chickpea genotypes (ILC 3279 and ChK 3226) endured severe drought, showing a high recovery ability after rehydration. However ILC 3279 despite solutes accumulation was more affected in photosynthetic activity and membrane integrity during water stress than ChK 3226. This genotype showed a higher protoplasmic resistance to dehydration as inferred

from a lower impact on membranes, evaluated by membrane leakage and lipid content. It showed an enhanced lipid synthesis under S1, which may improve plants adaptation to water stress, allowing the plant to cope with subsequent severe dehydration and to support a better recovery after rehydration. Such traits are in agreement with the better yield of ChK 3226 under drought, and may be used in breeding programs. Solutes accumulation did not seem to be a reliable indicator of better yield under drought.

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