

Physiological and Biochemical Responses of *Medicago truncatula* to Drought Stress

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It is frequently observed that drought stress decreased plant growth and induced cellular damages. However, the underlying physiological and biochemical mechanisms are not well understood. *Medicago truncatula* was subjected to drought stress by water withdrawing at mature stage and for one week. Drought stress reduced plant growth, inhibited photosynthesis, stomatal conductance and induced oxidative stress. Water stress induced changes e.g. it increased osmo-protectants (proline, glycine betaine), and the level of oxidative stress parameters (H_2O_2 and lipid peroxidation). In parallel with higher levels of H_2O_2 and MDA, there were increase in NADPH oxidase and lipoxygenase (LOX) activities in *Medicago truncatula*. Presumably as a consequence of the induction of H_2O_2 production, activation in some antioxidant defense components was observed (e.g. increased superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), peroxidase (POX) and glutathione reductase (GR)). Other antioxidant component were also significantly increased by drought stress.

Keyword: Drought, Legume, *Medicago truncatula*, Oxidative stress, Plant redox network.

Drought is a worldwide threat to plant growth and production, inducing many plant biochemical, molecular, and physiological perturbations. For instance, it down regulates rates of photosynthetic CO_2 assimilation and transpiration and decreases stomatal and mesophyll conductance (Lawlor and Tezara, 2009). Moreover, the combination of elevated temperature with drought could exacerbate the stress effect as some studies indicate a higher detrimental effect on plant growth and productivity than when each stress was applied individually (Savin and Nicolas, 1996). Stress-induced alterations in plant metabolism, generally increase reactive oxygen species levels (ROS) (Gill and Tuteja, 2010), ROS accumulation can damage plant cells at the level of nucleic acids, membrane lipids, chlorophyll and proteins (Foyer and Noctor, 2005). One ROS, hydrogen peroxide (H_2O_2) is not only an indicative of a harmful process, but also participates in many signaling, resistance and regulation mechanisms including, reinforcement of the cell wall (Dempsey and Klessig 1995), stomatal movement (Bright *et al.* 2006), photorespiration and photosynthesis (Foyer *et al.*, 2009),

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growth and development (Foreman *et al.* 2003) and hormonal signaling (Kwak *et al.*, 2006). Plants often minimize free radical damage by enhanced antioxidant defensive systems (Le Martret *et al.* 2011).

Accumulation of osmolytes, including polyols, free amino acids and soluble sugars, is a well-known plant response to water deficit (Szabados and Saviourè, 2010). Osmolytes are compatible solutes that adjust cellular osmotic potential, function as low molecular-weight chaperones, protect membranes and proteins, stabilize photosystem II, and protect against oxidative damage by scavenging ROS (Chen and Murata, 2008; Szabados and Saviourè, 2010). Increasing photosynthetic carbon assimilation and ATP synthesis (Johnson *et al.*, 2002). On the other hand, a significant reduction in proline and soluble sugar content was reported for wheat in combinations of drought and UV-C radiation.

Understanding and predicting future climate plant responses, is further complicated by significant 'species x future climate' interactions, *i.e.* species react differently to climate changes. For example, a competitive advantage of Legume-species over other species, under climate change was observed (Wand *et al.*, 1999), similarly, Hebeisen *et al.* (1997) has suggested that white clover could profit more than ryegrass. The competitive advantage of legumes arises from their ability to fix atmospheric N (Rogers *et al.* 2006).

The aim of the current research was, therefore, to analyze the drought response a legume species (*Medicago truncatula*), at the physiological (photosynthesis, stomatal conductance), and molecular (osmolytes, antioxidants, redox network) level. In addition, studying molecular mechanisms to drought stress, in a model species, may shed new light on some defense pathways.

Materials and Methods

Experimental set-up and plant harvest

A greenhouse experiment was conducted in 2014, at the University of Beni-Suef, Faculty of Science, Botany Department. Anitrogen fixing dicot (*Medicago truncatula* L.) was grown for 4 months in controlled growth chambers. The plants seeds were grown in pots filled with sandy soil (93.2% sand, 4.6% silt, 2.2% clay; field capacity 0.13 m³ m³; pH 7.6; total Kjeldahl-N 0.42 g kg⁻¹; 1% C in humus). During vegetative stage, water-deficit stress was induced by withdrawal of irrigation, and, depending upon how fast target species responded to drought (by observing yellowing, wilting or curling of leaves), it was applied for one week in *M. truncatula*. Aboveground biomass (fresh weight, FW) was collected by cutting plants 4 cm above the soil surface.

Photosynthesis, stomatal conductance and chlorophyll a

Light saturated photosynthetic rate (A_{sat} , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and stomatal conductance (g_s , $\mu\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) were determined (LI-COR LI-6400, LI-COR Inc., Lincoln, NE, USA) on the youngest fully expanded leaves. LI-COR leaf

chamber conditions were set at 385 or 630 ppm CO₂, and 23.5 or 26.5 °C (block temperature), according to the climate treatments, saturated photon flux density (1500 μmol.m⁻².s⁻¹), and ambient relative humidity. Sixteen replications per species were randomly measured. Chlorophyll “a” and carotenoid content were determined after acetone extraction and calculated according to Porra et al. (1989).

Proline and free amino acids

Plant shoots (200 mg FW) were homogenized in 2 ml of 3% (v/v) aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm for 20 min. Proline content was measured after toluene extraction using the ninhydrin reagent (Bates *et al.* 1973). Free amino acids (FAA) were determined in ethanolic extracts (0.1 ml 80% (v/v) ethanol, per 200 mg FW), and extraction in a ninhydrin-citrate-glycerol mixture (20 min, 100°C) (Lee and Takahashi 1966). Proline and glycine were used as standards respectively.

Glycine betaine

Plant material (200 mg FW) was ground by a MagNALyser in liquid nitrogen and extracted in deionized water (5 ml, 48 h, 25 °C). Quaternary ammonium compounds were measured according to Grieve and Grattan (1983), glycine betaine was used as the standard.

Soluble sugars

Plant shoots (200 mg FW) were ground by a MagNALyser in liquid nitrogen and interfering pigments were removed by extraction with 100% acetone. Sugar content was estimated with the anthrone reagent (Leyva et al., 2008), using a glucose standard curve.

Lipid peroxidation

Lipid peroxidation was determined on frozen shoot tissues, homogenized in 80% ethanol by mortar and pestle, using a thiobarbituric acid-malondialdehyde (TBA-MDA) assay (Hodges et al., 1999).

H₂O₂ concentration

H₂O₂ concentration was measured by the FOX1 method (Jiang et al., 1990), based on the peroxide-mediated oxidation of Fe²⁺, followed by reaction of Fe³⁺, with xylenol orange. Specificity for H₂O₂ was tested by eliminating H₂O₂ from the reaction mixture with catalase.

Total antioxidant capacity

Plant tissues (200 mg FW) were ground in liquid nitrogen and the antioxidants were extracted in 2ml of ice cold 80% ethanol. FRAP (ferric reducing/antioxidant power assay) reagent (0.3 M acetate buffer (pH3.6), 0.01 mM TPTZ in 0.04 mM HCl and 0.02 M FeCl₃.6H₂O) was mixed with the extract and measured at 600 nm using a microplate reader (Benzie & Strain, 1996). Trolox was used as standard.

Ascorbate, glutathione, homo-glutathione and their redox status

Ascorbate (ASC), glutathione (GSH) and homo-glutathione (hGSH) were determined by HPLC analysis. The redox status was calculated as the ratio of the reduced form to the total concentration of the antioxidant. In *M. truncatula*, GSH is largely replaced by hGSH, and the added concentrations (hGSH+GSH) are used throughout.

Tocopherols

Tocopherols were extracted with hexane using the MagNALyser. The dried extract (CentriVap concentrator, Labconco, Kansas, USA) was re-suspended in hexane, and tocopherols were separated and quantified by HPLC (Shimadzu, s Hertogenbosch, The Netherlands) (normal phase conditions, Particil Pac 5 μm column material, length 250 mm, i.d. 4.6 mm). Dimethyl tocol (DMT) was used as internal standard (5 ppm). Data were analyzed with Shimadzu Class VP 6.14 software.

Polyphenols and flavonoids

Polyphenols and flavonoids were extracted in 80% ethanol (v/v) and determined according to Zhang *et al.* (2006), and Chang *et al.* (2002), and with gallic acid and quercetin respectively as standards.

Enzyme assays

Lipoxygenase (LOX) was extracted in 50mMpotassium phosphate buffer (pH 7.0) and its activity was measured according to Axelrod *et al.* (1981). The standard assay mixture (0.2mL) consisted of 160 μL of sodium phosphate buffer (50 mM, pH 7.0); 20 μL of crude extract and 20 μL of a substrate emulsion (10 mM linoleic acid emulsified in 0.36% Tween-20). The formation of hydroperoxides was calculated by using extinction coefficient 25,000 $\text{M}^{-1}\text{cm}^{-1}$. One unit of enzyme was defined as the quantity that generates 1 mmol of conjugate diene per minute at 25°C.

NADPH oxidase was assayed according to Van Gestelen *et al.* (1997) and Sarath *et al.* (2007), after extraction in 50mMpotassium phosphate buffer (pH 7.0). Monoformazan concentrations were calculated using an extinction coefficient of 12.8 $\text{mM}^{-1}\text{cm}^{-1}$.

Ascorbate peroxidase (APX), mono-dehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were determined in microplates (Synergy Mx, Biotek Instruments Inc., Vermont, USA) (Murshed *et al.*, 2008). Peroxidase (POX) activity was determined by the oxidation of pyrogallol ($\epsilon_{430} = 2.47 \text{ mM}^{-1}\text{cm}^{-1}$) (Kumar and Khan, 1982). Superoxide dismutase (SOD) activity was determined by Dhindsa *et al.* (1981) by measuring the inhibition of NBT reduction at 560 nm. Catalase activity was assayed according to Aebi (1984) by monitoring the rate of decomposition of H_2O_2 at 240 nm. For all activities 100 mg frozen tissue was extracted with a MagNALyser, in 1 mL of extraction buffer (50 mM MES/KOH (pH 6.0), 4 mM KCl, 2 mM CaCl_2 and 1 mM ascorbic acid). In addition, measurements were scaled down for semi-high throughput using a micro-plate reader, and optimized

to obtain linear time and protein-concentration dependence.

Statistical analysis

Results were analyzed by one-way ANOVA, using SPSS 16.0 statistical software (COMPANY, CITY, COUNTRY), and significant differences between the means were determined by using the Duncan test ($P < 0.05$) ($n=16$).

Results

Effect of drought on plant growth, photosynthesis, stomatal conductance and chlorophyll content

Drought treatment reduced plant biomass, at the DW and FW level (Fig. 1 A&B). Light saturated photosynthesis (A_{sat}) was strongly inhibited ($P < 0.001$ compared to controls) (Fig. 2A). Similarly, strong reductions of stomatal conductance (g_s) were observed (Fig. 2B). Chlorophyll a content was also reduced by drought in *Medicago truncatula* (Fig. 2C).

Oxidative stress indicators

Oxidative stress levels were measured by H_2O_2 levels and lipid peroxidation. H_2O_2 levels were increased by drought (Fig. 3A). In parallel with the increase in H_2O_2 levels, also the NADPH oxidase activity increased under drought stress (Fig. 3B). Increases in MDA levels demonstrate that the drought treatment increases lipid peroxidation (Fig. 3C). The increases in MDA levels are largely matched by elevated levels of lipid peroxidase (LOX) activity (Fig. 3D).

Changes in osmolytes

Drought caused strong increases of proline in *Medicago truncatula* (Fig. 4A). In parallel to the proline levels, we investigated changes in overall free amino acid concentrations (Fig. 4B). Compared to changes in free amino acids, proline was less induced by drought. Glycine betaine concentrations increased under drought *Medicago truncatula* (Fig. 4C).

Antioxidant parameters: molecular antioxidant levels

As a tool to assess overall antioxidant changes, the measurement of Total Antioxidant Capacity (TAC) is commonly used. TAC increases as a consequence of drought stress (Fig. 5A). The TAC assay does not allow discrimination between changes in specific groups of antioxidant molecules. We therefore separately analyzed polyphenol, flavonoid and total tocopherol levels (Fig. 5B-D). In *Medicago truncatula*, drought increased the levels of polyphenols, flavonoids and tocopherols, although to different extents, (Fig. 5B-D).

Antioxidant parameters: ROS scavenging enzymes SOD, POX, APX, GPX and CAT

Superoxide dismutase (SOD) activity was induced by drought in *Medicago truncatula* (Fig. 6A). The responses of hydrogen peroxide scavenging enzymes to stress conditions were recorded. Peroxidase and Catalase (CAT) activity increased under drought stress (Fig. 6B). General peroxidase (POX) and ascorbate oxidase (APX) activity increases were observed (Fig. 6C and D). Similar

to CAT, glutathione peroxidase (GPX) activity also increased in drought (Fig. 6E).

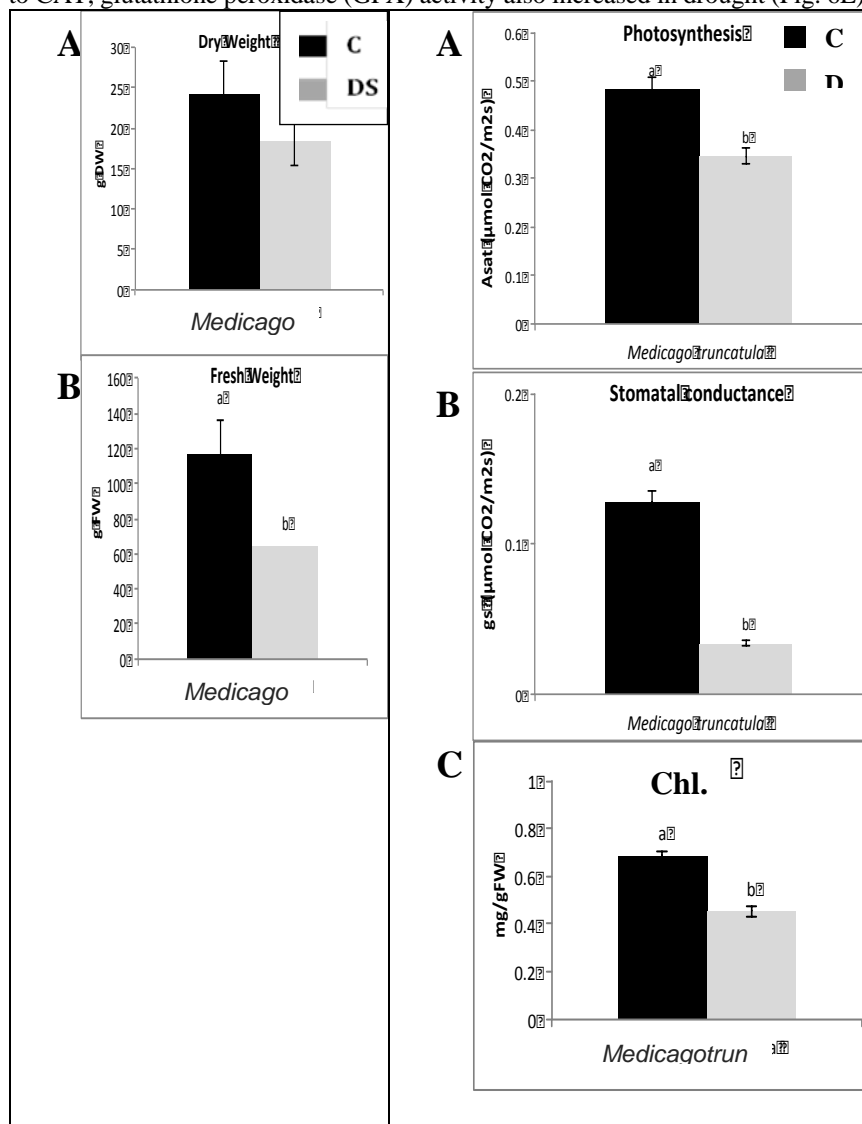


Fig. 1. Above ground biomass of *Medicago truncatula* under drought stress (Ds) compared with control (C); (A) dry weight, (B) fresh weight. Different letters in the graph represents the significant differences between the four treatments (Duncan test; $P < 0.05$; $n=4$).

Fig. 2. Photosynthesis, stomatal conductance and chlorophyll "a" content of *Medicago truncatula* under drought stress (Ds) compared with control (C); (A) light saturated net CO_2 assimilation rate (Asat), (B) stomatal conductance (gs), (C) chlorophyll "a" content. Different letters in the graph represents the significant differences between the four treatments (Duncan test; $P < 0.05$; $n=4$).

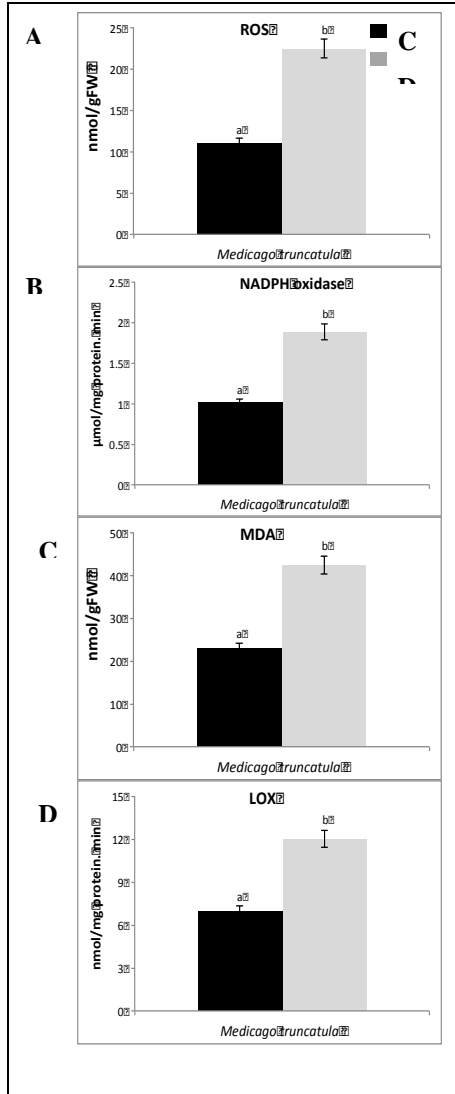


Fig. 3. Oxidative stress indicators of *Medicago truncatula* under drought stress (Ds) compared with control (C); (A) hydrogen peroxide, (B) NADPH oxidase activity, (C) lipid peroxidation detected as malondialdehyde (MDA), (D) lipoxigenase activity (LOX). Different letters in the graph represents the significant differences between the four treatments (Duncan test; P < 0.05; (n=4).

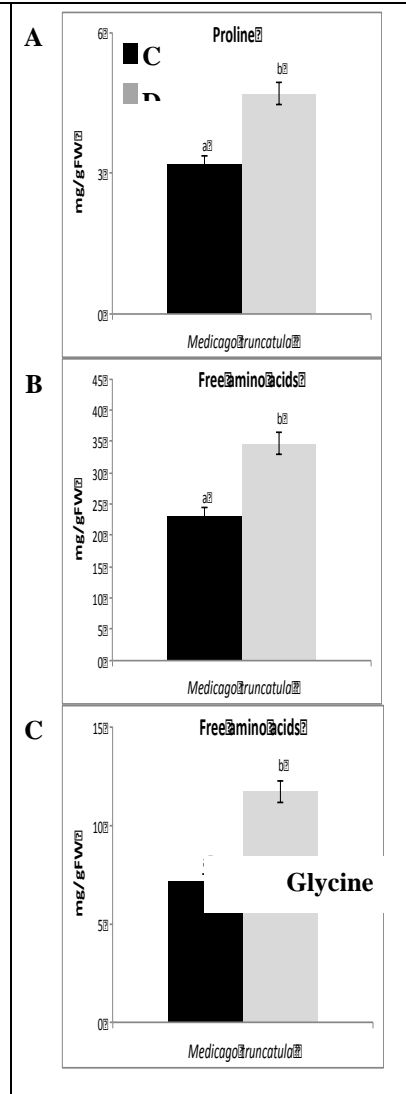


Fig. 4. Osmolytes and glycine serine ratio of *Medicago truncatula* under drought stress (Ds) compared with control (C); (A) proline, (B) free amino acids, (C) glycine betaine.

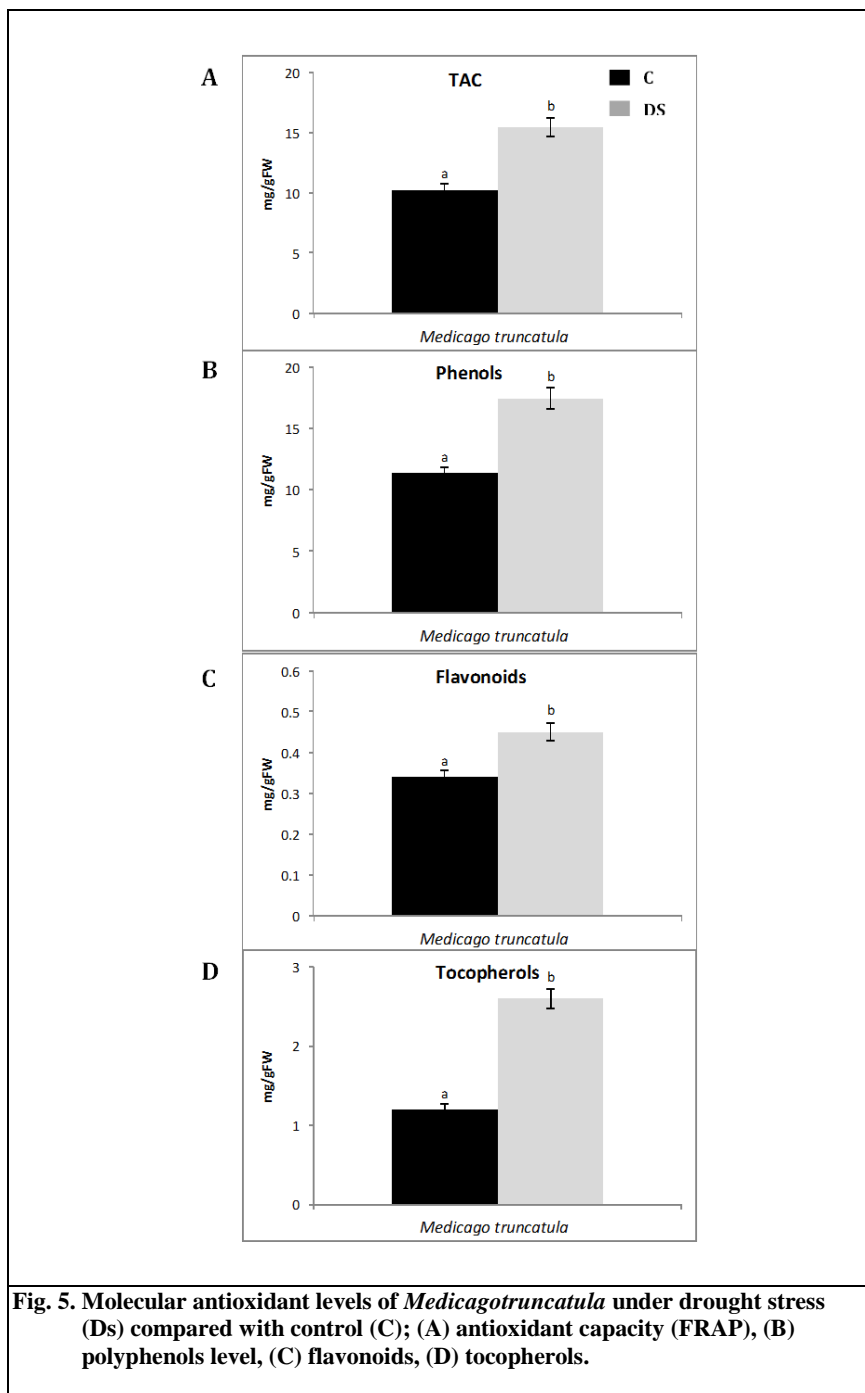
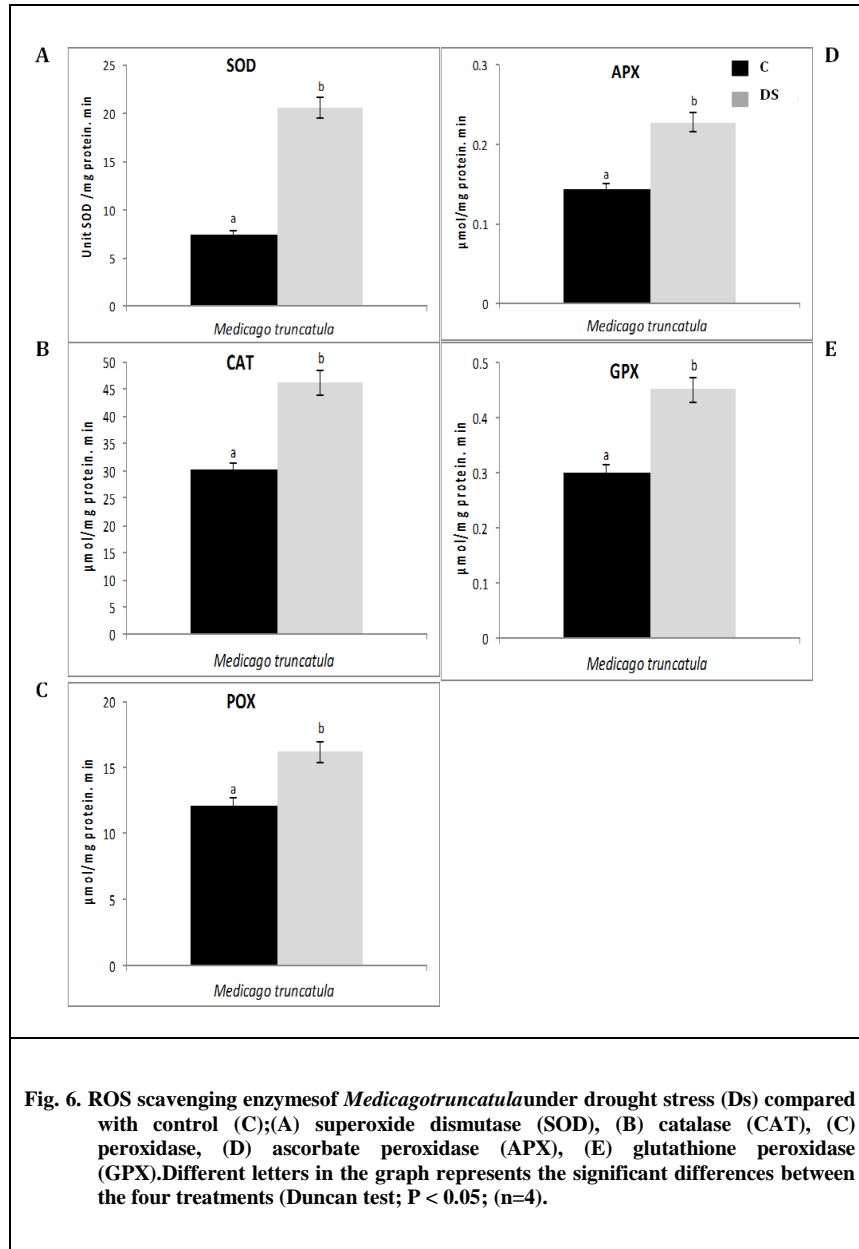


Fig. 5. Molecular antioxidant levels of *Medicago truncatula* under drought stress (Ds) compared with control (C); (A) antioxidant capacity (FRAP), (B) polyphenols level, (C) flavonoids, (D) tocopherols.



Antioxidant parameters: ascorbate-glutathione cycle

One well-documented antioxidant defense system involves reduced ascorbate (ASC) and glutathione (GSH) as principal electron donors, hydrogen peroxide scavenging enzymes (APX, GPX), and reductant recycling enzymes (GSH-dependent DHA reductase (DHAR), MDHAR, glutathione reductase (GR) (Foyer and Noctor 2005; Potters *et al* 2004). The redox status of ASC and GSH, *i.e.* the relative balance of the reduced and oxidized molecules, is often considered an indicator for oxidative stress conditions.

Levels of ASC as well as the ASC redox status, were hardly affected by the drought, (Figure 7A and B). There was a tendency of ASC redox status to decrease. The ascorbate redox status was generally high (70-90%), in control conditions, and decreased (strongly) to about 40% under drought. GSH levels increased by the drought in *Medicago truncatula*. The glutathione redox status in stressed conditions was low in *Medicago truncatula* (27%) (Figure 7D). The activity of the GSH-dependent ASC recycling enzyme DHAR increased under drought, (Figure 7C). Changes in GSH recycling GR activity, induced by stress were similar to the changes in GSH levels (Figure 7F).

Discussion

Photosynthesis and stomatal conductance

The present study clearly showed the inhibitory effect of drought on A_{sat} and g_s . In the same way, Shah and Paulsen 2003, Xu and Zhou 2006, stated that drought application resulted in more detrimental effect comparing with its effect separately. Although some studies stated that the limitations of photosynthesis under most drought conditions are mainly related to mesophyll conductance (Flexas *et al.*, 2006). But others suggested that the changes in photosynthesis under stress are not directly related to stomatal conductance (Lodge *et al.*, 2001), particularly with severely combined stresses (Chaves *et al.* 2003). Similarly, we found unparalleled changes in A_{sat} and g_s condition which proposed the non-stomatal limitations of the photosynthesis (A_{sat}). Since, alteration in photosynthetic capacity can be attributed to photo-inhibition, oxidative damage to protein and Chls. and alteration in Rubisco activity or reallocation of leaf nitrogen content (Smirnoff 1998, Flexas and Medrano, 2002).

Changes in osmotic protectants

Proline and glycine betaine are known to be stress-related compounds (Chen & Murata, 2008) and their extent of induction depended on plant species and the severity of stress (Goyal and Asthir 2010, Chen & Murata 2011). In our study, overall drought caused considerable inductions of proline and glycine betaine. Accumulation of large amounts of osmolytes is an adaptive response and a protection mechanism against drought promoted cellular damage, by detoxification of reactive oxygen species, stabilization of proteins and protein complexes, protecting chloroplast and photosynthesis system II (PSII), and indirectly interacting with phosphatidylcholine moieties of membranes to alter their thermodynamic properties and as signaling/regulatory molecules (Chen & Murata, 2008; Szabados and Savaresi, 2010). For example, *Lotus corniculatus* cv. San Gabriel under drought and salt (Borsani *et al.* 1999) and sugarcane under heat stress (Wahid and Close 2007) were reported to accumulate large amounts of proline or GB.

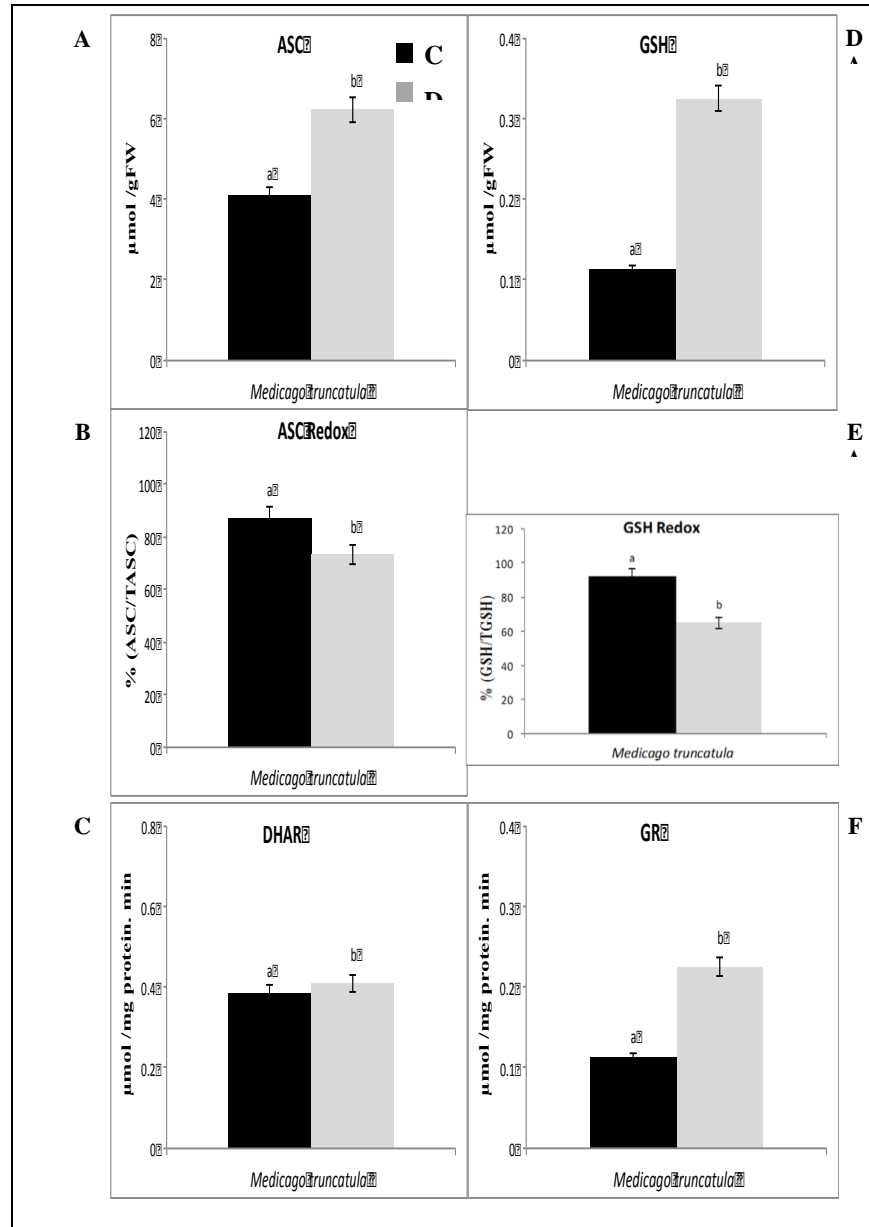


Fig. 7. Ascorbate-glutathione cycle of *Medicago truncatula* under drought stress (Ds) compared with control (C); (A) ascorbate, (B) ascorbate redox status (ASC/totalASC), (C) dehydroascorbate reductase (DHAR), (D) glutathione, (E) glutathione redox status (GSH/totalGSH), (F) glutathione reductase (GR). Different letters in the graph represents the significant differences between the four treatments (Duncan test; $P < 0.05$; $n=4$).

Oxidative stress / antioxidants

Apart from changes in osmo-protectants, drought typically also results in oxidative stress responses (Erice *et al.* 2007; Cruz de Carvalho 2008). Generally, these results confirm the occurrence of oxidative cellular damage, as well as the induction of antioxidant responses by drought demonstrating that plants experience oxidative stress. Lipid peroxidation (MDA) as one of stress damage markers was increased with drought. The increases of MDA matched well with rise of H₂O₂ by drought condition which could be an explanation for high destructive oxidative processes (eg., MDA) (Salazar-Parra *et al.* 2012). Moreover, Schwanz and Polle (1998) and Erice *et al.* (2007) attributed the low rate of oxidative damage to the decrease in ROS formation which resulting from an enhanced use of reductant for assimilation in photosynthesis and a reduced photorespiration.

Similar increases by drought are observed in total antioxidant capacity, in prominent classes of antioxidant molecules, the flavonoids and polyphenols, and in the lipid-phase antioxidants, the tocopherols. Induction of ROS scavenging as an adaptive response to stressful condition to minimize free radicals damage was also observed in many studies (Mittler *et al.* 2004; Gill and Tuteja 2010). The content of the water soluble and non soluble antioxidants increased under drought stresses and also increases under elevated-temperature conditions (Borsand Michel 2002), which depend strongly on the species, and plant growth stages (Munné-Bosch *et al.* 2013). Remarkably, despite increases in various antioxidant molecules, including membrane-associated tocopherols, the increased level of lipid peroxidation demonstrates that these increases are insufficient to effectively protect the plant membranes where ROS production exceeded the capacity of the antioxidative systems to remove them (Smirnov 1993; Gill & Tuteja, 2010)

ASC / GSH - cycle

The ascorbate-glutathione cycle, constitutes an important antioxidant system in plants. Reduced ascorbate (ASC) is oxidized by APX-mediated H₂O₂-scavenging reactions, and regenerated by mono-dehydroascorbate reductase (MDHAR) and/or dehydroascorbate reductase (DHAR), at the expense of NADPH and reduced glutathione respectively (Potters *et al.* 2004; Foyer and Noctor 2005). These enzymes are therefore believed to determine cellular ASC levels as well as the ascorbate redox status.

However, quite in contrast with the other parameters we studied, the patterns of changes induced by drought in ASC, APX, MDHAR and DHAR. For example, in *Medicago lupulina*, the decreasing ASC levels with drought correlate to increased APX levels (consumption), and decreasing MDHAR activities (regeneration). Yet, at the same time, DHAR activities (regeneration) are also increased, which should result in higher ASC levels. As a result of this heterogeneity, it is very difficult to correlate the changes in ASC levels to changes in particular enzymes. There was no correlation between ASC/GSH

content and their enzymes also was recorded in other studies for instance, Erice *et al* (2007), stated that GSH increased and ASC decreased while GR decreased and APX increased. The increase in ASC content also combined by increased in APX. Jimenez *et al* (1998) also found the same uncorrelated pattern in pea leaves, where APX and MDHAR strongly decreased but ASC also decreased and GR decreased while GSH increased. Hernandez *et al* (1999) observed the same state in pea under High CO₂ (Decreasing the ASC with increasing DHAR and MDHAR while APX slightly up regulated).

Conclusion

Drought stress significantly reduced photosynthesis and hence less biomass production of *Medicago truncatula*. It also induced the antioxidant defense system, however oxidative stress was still increased. Induced oxidative stress was mirrored by high H₂O₂ levels and that was accompanied by a significant induction in the most of antioxidants e.g., SOD, CAT, GPX, POX and GR and ASC and GSH.

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(Received 27/6/2016;
accepted 6/3/2017)

الاستجابات الفسيولوجية والبيوكيميائية للفصّة البرميلية تجاه إجهاد الجفاف

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كثيراً ما يلاحظ إن إجهاد الجفاف يؤدي إلى انخفاض نمو النبات و العديد من الأضرار الخلوية الناتجة عنه. ومع ذلك، لا يتضح الفهم الكامل للآليات الفسيولوجية والبيوكيميائية الأساسية المرتبطة به. في هذا البحث، تم تعريض نبات الفصّة البرميلية (الفصيلة البقولية) لإجهاد الجفاف عن طريق سحب المياه في مرحلة النضج ولمدة أسبوع واحد. وقد أدى إجهاد الجفاف لانخفاض نمو النبات، مع تثبيط التمثيل الضوئي، و حركة الغازات من و إلى الثغور والأكسدة التي سببها. ومن التغيرات الناجمة أيضاً عن الإجهاد المائي، على سبيل المثال زيادة الوقاية من التناضح عن طريق زيادة المحتوى من البرولين و البيتاين ومستوى المعلمات الأكسدة (أول أكسيد الهيدروجين و بيروكسيد الدهون). و بالتوازي، يصاحب المستويات العالية من أول أكسيد الهيدروجين MDA، نشاط واضح لكلاً من أكسجيناز الدهني و NADPH. و نتيجة لزيادة إنتاج أول أكسيد الهيدروجين، لوحظ ظهور نشاط واضح في بعض مكونات الدفاع لمضادات الأكسدة (على سبيل المثال زيادة السوبر أكسي ديس ميتوايز (SOD)، الكاتاليز (CAT)، الجلوتاثيون بيروكسيداز (GPX)، البيروكسيداز (POX) و الجلوتاثيون المختزل (GR). و تبع ذلك زيادة ملحوظة في مكونات أخرى لمضادات الأكسدة نتيجة لإجهاد الجفاف (دورة أسكورات الجلوتاثيون).