

# Water extraction under terminal drought explains the genotypic differences in yield, not the anti-oxidant changes in leaves of pearl millet (*Pennisetum glaucum*)

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**Abstract.** Pearl millet (*Pennisetum glaucum* (L.) R.Br.) is a resilient crop suiting the harshest conditions of the semi-arid tropics, in which we assessed possible relationships between crop tolerance, anti-oxidative enzyme activity and plant/soil water status. Biochemical acclimation and cell homeostasis traits have been proposed as critical for the drought tolerance of crops, but their limited practical application in breeding so far suggests that the role of biochemical acclimation for drought tolerance is still unclear. Previous research may have been of limited value because it has not approached biochemical acclimation from the angle of plant water relations. Four pearl millet genotypes, contrasting for terminal drought tolerance, were evaluated (sensitive H77/833–2, tolerant PRLT2/89–33 and two near isogenic lines carrying a terminal drought tolerance quantitative trait locus) under water-stress (WS) and well-watered (WW) conditions in a lysimetric system that simulates field-like conditions. We assessed the genotypic variation and relationship between photosynthetic pigments (chlorophylls *a* and *b* and carotenoids), antioxidative isoenzymatic spectrum (superoxide dismutase, ascorbate peroxidase and catalase), physiological traits (soil moisture available, normalised transpiration, stay-green score and water extraction) and biomass and yield. Biochemical traits investigated were tightly related among each other under WS conditions but not under WW conditions. Two major ascorbate peroxidase isoforms (APX6&7), whose variation in both water regimes reflected the presence/absence of the drought tolerance quantitative trait locus, were identified, but these did not relate to yield. Both, yield and biochemical traits under terminal drought stress were closely related to the traits linked to plant/soil water status (soil moisture available, normalised transpiration, stay-green score and water extraction), whereas yield and the biochemical indicators were not correlated, except for one. It is concluded that there is no direct effect of biochemical traits on yield parameters since both are consequences of soil-plant water status and their putative relation appear to be secondary – through plant/soil water status.

**Additional keywords:** ascorbate peroxidase, catalase, soil water, stay-green, superoxide dismutase, water extraction.

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## Introduction

From one point of view, there is a conventional knowledge that crop water usage at key stages is critical for drought adaptation in the field conditions (e.g. Hammer *et al.* 2006; Manschadi *et al.* 2006; Passioura 2006; Zaman-Allah *et al.* 2011); from another point of view, traits related to plants biochemical acclimation and cell homeostasis (e.g. osmolytes, antioxidative enzymes, photosynthetic pigments) have long been proposed as critical for the drought tolerance of crops (e.g. Sairam and Srivastava 2001; Fazeli *et al.* 2007) and have been the object of many studies (see review by Cruz de Carvalho 2008), including substantial investments in development of transgenics to harness these traits (see review by Bhatnagar-Mathur *et al.* 2008). But how do the two fit together? To our knowledge, there has been little side-by-side investigation to compare the importance of both aspects for drought adaptation. This is in part because of practical limitations in experimental systems to reliably and precisely assess plant/

soil water status along with biochemical measurement and agronomically important parameters. To date, no evidence of success using these biochemical traits in breeding has been reported, which might be at least partially due to an incomplete understanding of the basis of drought tolerance mechanisms in natural field conditions (see review by Tardieu 2011). The over-emphasis on drought acclimation processes (i.e. internal plant biochemical adjustment to tissues water deficit) for the crops drought tolerance simply appears to overlook one critical factor: water. Recent work on pearl millet showed that constitutive conservative use of water (Kholová *et al.* 2010a, 2010b, 2012) rather than plasticity of biochemical pathways upon drought exposure (Kholová *et al.* 2011) are important for crop success in terminal drought conditions. The importance of water use related processes for drought tolerance are also emphasised in other species; e.g. chickpea (Zaman-Allah *et al.* 2011), sorghum (Hammer *et al.* 2006), cowpea (Belko *et al.* 2012). Here, our

hypothesis is that high yield under terminal drought stress, taking the case of pearl millet, would rather be related to differences in water usage and that possible difference in biochemical acclimation under stress would come only as consequence of plant/soil water status.

In an earlier study of biochemical acclimation mechanisms (Kholová *et al.* 2011), the anti-oxidative enzymes machinery (APX, SOD, CAT isoenzymatic activities) and their relations to the photosynthetic pigments (chlorophylls *a*, *b* and carotenoids) contents, were compared in materials introgressed or not with a major terminal drought tolerance quantitative trait loci (DT-QTL; Yadav *et al.* 2002). The majority of these traits were not associated with the DT-QTL presence. Nonetheless, these experiments were conducted in the glasshouse pot culture with regulated drought imposition (plants were maintained at similar soil moisture levels), where long-term plant water management related traits as well as agronomically important traits could not be reliably assessed and compared with behaviour of the crop in the field conditions. Here, the pearl millet materials are evaluated in a lysimetric facility, i.e. a set of long and large tubes filled with Alfisol that are set up under natural conditions and mimic closely the field conditions in terms of soil volume and surface area available to each plant (Vadez *et al.* 2011). This system allows the imposition of naturally occurring stress treatments (e.g. Ratnakumar and Vadez 2011; Zaman-Allah *et al.* 2011) and precise assessment of plant water use and soil moisture available through the entire cropping cycle, along with yield assessment at maturity, and the possibility to sample leaf tissue for biochemical measurement. In short, it allows for precise measurement of traits (agronomical, physiological, biochemical) that were not measured side-by-side in earlier studies. Here, we test the link between agronomical traits, biochemical traits and their relation to indicators of plant and soil water status under terminal drought stress.

Therefore, the aim of this work was to compare agronomically important traits with biochemical drought acclimation processes, their relations to plant/soil water status and their importance in a terminal drought tolerance in pearl millet; specifically to: (i) assess agronomically important characteristics along with the anti-oxidative enzymes activities and pigments contents in plants introgressed or not with a DT-QTL and exposed to terminal drought conditions in field-like situation; and (ii) analyse the relationships between these biochemical traits and characteristics reflecting the soil-plant moisture status.

## Materials and methods

The experiment was conducted during the summer season (5 February to 15 April 2010) at the ICRISAT lysimetric facility at Patancheru (Vadez *et al.* 2008), India: latitude, 17°30'N; longitude, 78°16'E, altitude 549 m. The maximum and minimum temperatures during the experimental period averaged 35.8 and 19.9°C, respectively: the minimum and maximum RH averaged 26.3 and 74.9% respectively.

### Plant material

Two pearl millet (*Pennisetum glaucum* (L.) R. Br.) genotypes and two DT-QTL-introgressed lines (NILs); PRLT2/89-33 (tolerant parent) and H77/833-2 (sensitive parent) and ICMR

01029, ICMR 02042 (NILs) were selected from previous experiments (Kholová *et al.* 2010a). Work was conducted on test-cross hybrids of these genotypes developed by crossing the inbred parental lines and QTL-NILs to the male sterile line tester 843A (for the reasons described by Yadav *et al.* 2002). The two selected QTL-NILs had higher yield under terminal drought in field conditions (ICMR 01029) and lysimetric conditions (ICMR 02042, V. Vadez, J. Kholová, R. S. Yadav and C. T. Hash, unpubl. data) than the recurrent parent H77/833-2 (Serraj *et al.* 2005). Detailed description of plant material is elsewhere (Kholová *et al.* 2011).

### Plant growth in lysimetric systems

#### Soil filling and growth conditions of the lysimeters

Plants were grown in lysimeters, i.e. PVC tube of 25 cm diameter and 2.0 m length, filled with Alfisol as described previously (Vadez *et al.* 2008, 2011). The Alfisol used to initially fill the tubes had been collected at the ICRISAT farm, sieved in particles smaller than 1 cm and fertilised with di-ammonium phosphate and muriate of potash (both 200 mg kg<sup>-1</sup> of soil) and farm manure (at 1:25, w/w). The lysimeters had been cropped several times before running this experiment, so that we consider that the soil profile was similar to a soil profile in the field and not like a freshly packed soil as is usually the case in potted trials. Subsequent weighing of the cylinders indicated that the weight of all cylinders at field capacity was within a narrow range (typically between 163 and 165 kg).

#### Space arrangement of the lysimeters and weighing procedure

The top of the cylinders was equipped with a metal collar and rings that allowed the cylinder to be lifted. Weighing of the cylinders was done by lifting the cylinders with a block-chained pulley; and an S-type load cell (Mettler-Toledo, Geneva, Switzerland) was inserted between the rings of the cylinder and the pulley. The scale, of 200 kg capacity allowed repeated-measurements with reasonable accuracy of 20 g on each weighing. The lysimeters were separated from one another by a distance of ~5 cm. Therefore, the millet crop was planted at a density of ~11 plants m<sup>-2</sup> (which represents slightly lower plant population compared with local field practice i.e. around 15 plants m<sup>-2</sup>). The tubes were arranged in one of the available trenches of 2 m depth and 1.75 m width. Each trench was separated by a 20-cm concrete wall. We used an alpha-lattice design to help separate possible edge effects.

### Plant management and drought stress imposition

Two seeds were planted in each of three hills on 5 February, then thinned to two seedlings per cylinder at 7 days after sowing (DAS) and then to one plant per cylinder at 14 DAS. All plants were kept under fully irrigated conditions until 25 DAS by receiving equal amount of water per cylinder; i.e. 500 mL twice a week until two weeks after sowing and then on alternate days until 25 DAS. After the regular irrigation at 25 DAS, the cylinders were covered with a 2-cm layer of low density polyethylene beads, which prevented >90% of soil evaporation (data not shown). Weighing of the cylinders was done at 27, 32, 38, 45, 52, 59 and 69 DAS.

To maintain the well-watered (WW) plants fully irrigated and to avoid water drainage after irrigation, the watering to the WW plants was done when the cylinder weight, at the time of weighing, had fallen below 2 L from the weight at field capacity. This way, the weekly re-watering brought back the plants to ~85–90% of the field capacity (there is at least 13 L of water available in each tube). In any week, the maximum transpiration of WW plant did not exceed 4.5 L and so the soil of WW plant never fell below 50% field capacity. These conditions ensured that the soil was kept sufficiently wet to allow maximum growth while preventing drainage at the bottom during re-waterings. The drought stress (DS) treatment received regular irrigation until 25 DAS, a last irrigation of 2 L per plant at 35 DAS, given between booting and flowering stage and equivalent to a 50 mm irrigation under field conditions and then no more irrigation from 35 DAS until maturity. From this point onwards the plants gradually entered into a soil moisture deficit that depended on their transpiration demand. This system also assured, all DS plants had the very same amount of water available for completion of their life cycle and therefore closely resembled the field situation.

Water extraction (WE; i.e. plants transpiration) was calculated for intervals between weightings until maturity (see above). The transpiration of stressed plants was compared with the control ones and expressed as normalised transpiration ratio (NTR, for details see Kholová *et al.* 2010a; Vadez and Sinclair 2001). The regular weighing further allowed the estimation of the fraction of the transpirable soil water (FTSW). The FTSW values represented the portion of remaining soil moisture available for transpiration during the course of the experiment and were used as our indicator of stress. FTSW of day  $n$  was calculated as:

$$\frac{(\text{cylinder weight of day } n - \text{final cylinder weight})}{(\text{initial cylinder weight} - \text{final cylinder weight})} \quad (1)$$

At maturity, the plants were harvested and dried for 3 days in a forced-air oven set at 70°C to estimate biomass DW (BDW, i.e. stover yield or plant DW excluding the panicle weight). Panicles were threshed afterwards to determine grain yield.

#### Sample collection for biochemical analysis and stay green scoring

##### Monitoring of drought imposition, sampling for biochemical assays and stay-green score

The response of biochemical traits to progressive exposure to water deficit was assessed twice during reproductive stage (at 52 and 59 DAS, i.e. 15 and 22 days after flowering). The samples for estimation of photosynthetic pigments contents were collected at two time points where the relative transpiration of the WS plants had dropped compared with their WW control; At 52 DAS (further referred as mild stress – MS, with a corresponding well-watered control, WW1), the fraction of transpirable soil water (FTSW), our index of stress and a measure of soil volumetric water content remaining available for transpiration, varied between 22 and 28% in all genotypes and the normalised transpiration ratio (NTR) (i.e. the transpiration of DS plants relative to that in WW plants) varied between 42 and 57%. At 59 DAS (further referred as severe stress – SS; corresponding control WW2), FTSW varied between 7 and 10% in all genotypes and NTR varied between 14 and 21%.

The collected samples of leaf tissue for the study of photosynthetic pigments content (chlorophyll *a*, *b* and carotenoids) were stored in a deep-freezer (–80°C) until the analysis. The following mornings (30 March and 6 April; i.e. 53 and 60 DAS) another set of leaf samples was collected for an immediate assessment of anti-oxidative enzymes activities. For the assessment of photosynthetic pigments and anti-oxidative enzymes the methodology described by Kholová *et al.* (2011) was followed with slight modifications. Here we used picture analysing program package QuantityOne Software (Bio-Rad, Hercules, CA, USA), ver. 4.0 using the option; ‘relative quantity’ which normalise the sum of band light intensity to the average of the full line intensity (this assure reliable cross-comparison between gels produced at different sampling times). These normalised values were later used for further analysis. The modification in assessment of isoenzymatic activities of Cu/ZnSOD 1 and 2 was also necessary because during the second estimation we failed to separate these two isoforms (Fig. 1a–c), therefore, even in the first assessment these were summed together to assure reliable comparison between MS and SS.

In addition, plants were visually scored for stay-green phenotype (on a scale 0–5, where 5 indicates a green plant and 0 is a fully dried plant) on 31 March and 12 April (54 and 66 DAS).

#### Statistical analysis

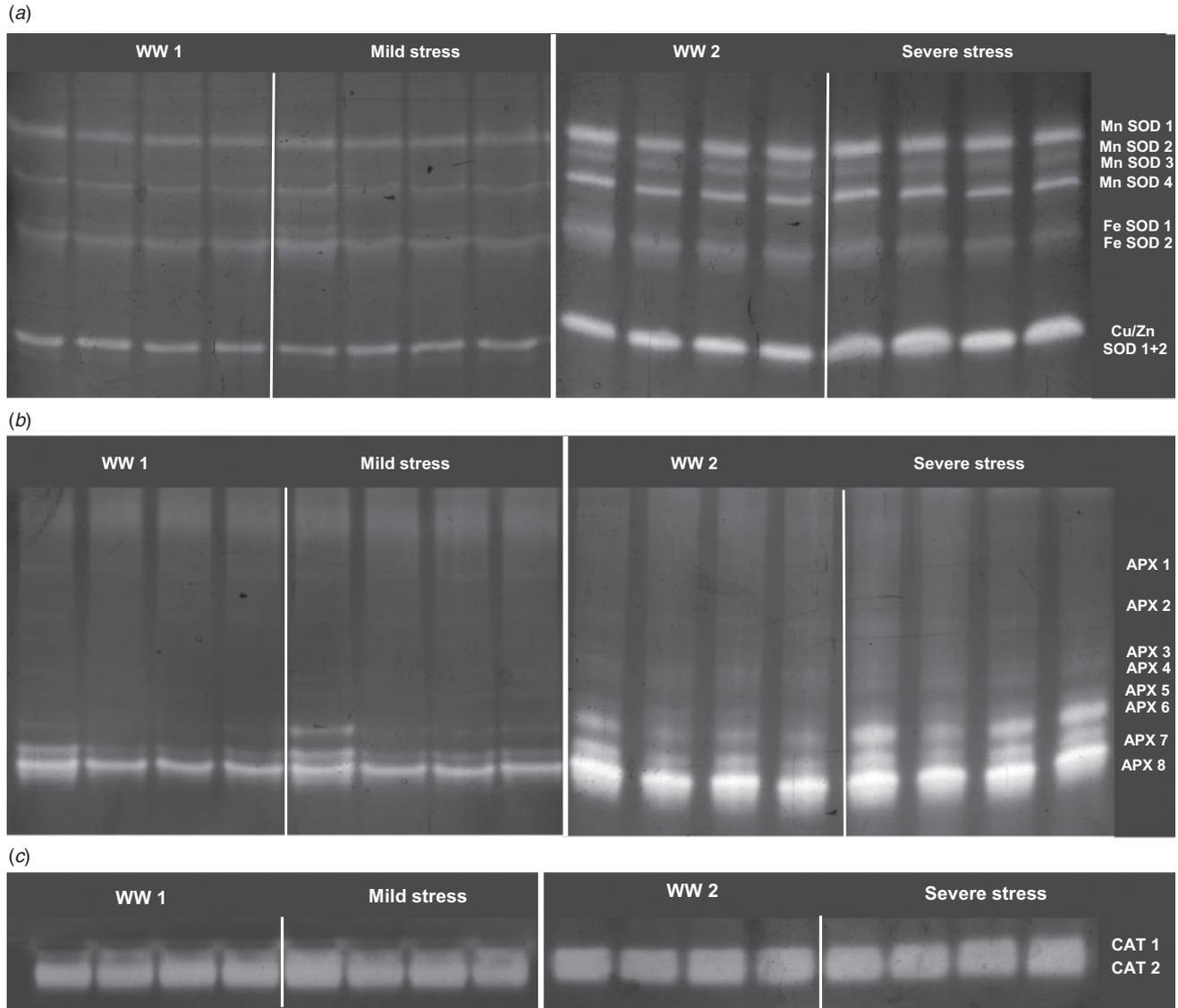
We used a completely randomised experimental design with four water treatments (WW1, WW2, MS and SS) as main factors and genotypes as subfactors, with five replications. ANOVA analyses were conducted with the statistical program package CoStat ver. 6.204 (CoHort Software, Monterey, CA, USA). Two-way ANOVA was conducted to compare differences in replicated data on photosynthetic pigments, biomass, yield, FTSW, NTR and STG across genotypes and water treatments. One-way ANOVA was conducted to compare the effect of drought treatments on enzymatic activities between WW1 and MS and between WW2 and SS across genotypes. The block ANOVA design (in which MS and SS, WW1 and WW2 were considered as separate blocks) was used to compare the overall effect of stress treatments on enzymatic activities. The means were further compared using a least significance difference (LSD) test. Simple correlation analysis between all parameters was performed between means of both stress treatments together and both control treatments together for traits which were assessed twice (during MS and SS). The correlation of all parameters with yield and stover which were estimated only once at the end of experiment was performed within WW and WS using Kendall–Spearman test (to avoid different data distribution within both samplings (MS, SS)).

## Results

### Analysis of physiological and agronomic traits

#### Yield and stover

The drought treatment decreased grain yield by ~50% across genotypes (Table 1). Under WW conditions, one-way ANOVA showed that sensitive genotype (H77/833–2) and both NILs (ICMR 01029 and ICMR 02042) had higher grain yield than the tolerant parent (PRLT2/89–33). In contrast, under WS



**Fig. 1.** The activity staining of (a) superoxide dismutase (SOD); (b) ascorbate peroxidase (APX); and (c) catalase (CAT) isoenzymes. The activities were assessed at two time points; 52 (mild stress) and 59 (severe stress) days after sowing (DAS) in plants grown in lysimetric system. The differentiation and quantitative measurements of isoenzymatic activities was conducted with QuantityOne Software (ver. 4.0) and the results of this analysis are shown in Table 1. The order of the pearl millet genotypes is PRLT2/89–33 (tolerant parent), H77/833–2 (sensitive parent), ICMR 01029, ICMR 02042 from the left for each treatment; i.e. well-watered control (WW1) and mild stress at 52DAS; well-watered control (WW2) and severe stress at 59 DAS.

conditions, sensitive H77/833–2 had lower yield than tolerant PRLT2/89–33 and ICMR 02042 (Table 1; Fig. 2).

Drought treatment also reduced BDW across genotypes but there were no significant genotypic differences in WW treatment. Under WS conditions, stover yield was significantly higher in sensitive parental genotype (H77/833–2) than in NILs and PRLT2/89–33 (Table 1).

*Water extraction (WE), fraction of transpirable soil water (FTSW), normalised transpiration ratio (NTR) and stay-green score (STG)*

ANOVA statistics didn't differentiate WE of genotypes between 45–52 and 52–59 DAS. However, NILs had a higher

FTSW at both sampling dates (52 and 59 DAS) than in H77/833–2 (Table 1). There were also significant differences in NTR (Table 1; Fig. 2), likely related to the differences in soil water availability (FTSW). A one-way ANOVA showed that sensitive genotype H77/833–2 had lower NTR than PRLT2/89–33 and one NIL in the medium stress treatment (MS). The block ANOVA confirmed that NTR was higher in PRLT2/89–33 and NILs than in H77/833–2 across both stages (Table 1). The block ANOVA showed lower stay-green scores in the sensitive parent (H77/833–2, which appeared dryer) compared with tolerant parental line and both NILs (Table 1; Fig. 2). The one way ANOVA showed that these differences were more pronounced in MS conditions. We interpret these

**Table 1.** Analysis of grain yield, stover yield, normalised transpiration ratio (NTR), fraction of transpirable soil water (FTSW), stay-green score (STG) and water extracted (WE) between 45–52 and 52–59 days after sowing (DAS)

Replicated data were analysed using one-way ANOVA and NTR, FTSW, STG and WE were further compared using block ANOVA design (as blocks were considered observations during mild stress (MS) and severe stress (SS)). Means were further compared using and *t*-test, i.e. lower case letters for one-way ANOVA and letters in NTR, FTSW and STG columns for block ANOVA; different letters indicate a significant difference ( $P < 0.05$ ) of means for particular trait between treatments and *vice versa*. With each mean,  $\pm$  s.e. is shown as measure of variability

Treatment	Genotype	Grain yield (g)	Stover yield (g)	NTR MS	NTR SS	NTR block	FTSW MS	FTSW SS	FTSW block	STG 54 DAS	STG 66 DAS	STG block	WE (g) 45–52 DAS	WE (g) 52–59 DAS	WE (g) block
WS	PRLT2/89–33	15.73 $\pm$ 1.18a	30.41 $\pm$ 0.90b	0.46 $\pm$ 0.035a	0.16 $\pm$ 0.03a	a	0.24 $\pm$ 0.02a	0.07 $\pm$ 0.01a	ab	3.22 $\pm$ 0.21a	1.11 $\pm$ 0.33ab	a	2469 $\pm$ 109a	844 $\pm$ 130a	a
WS	H77/833–2	12.06 $\pm$ 0.69b	35.06 $\pm$ 0.96a	0.42 $\pm$ 0.042b	0.14 $\pm$ 0.02a	b	0.22 $\pm$ 0.02a	0.07 $\pm$ 0.01a	b	2.33 $\pm$ 0.45b	0.67 $\pm$ 0.22b	b	2442 $\pm$ 194a	762 $\pm$ 139a	a
WS	ICMR 01029	14.92 $\pm$ 0.82ab	29.97 $\pm$ 1.47b	0.44 $\pm$ 0.032a	0.18 $\pm$ 0.03a	ab	0.28 $\pm$ 0.03a	0.11 $\pm$ 0.02a	a	3.2 $\pm$ 0.39a	1.9 $\pm$ 0.43a	a	2318 $\pm$ 107a	969 $\pm$ 129a	a
WS	ICMR 02042	16.49 $\pm$ 1.4a	29.70 $\pm$ 1.64b	0.49 $\pm$ 0.032a	0.19 $\pm$ 0.03a	a	0.26 $\pm$ 0.02a	0.09 $\pm$ 0.02a	a	3.2 $\pm$ 0.39a	1.2 $\pm$ 0.39ab	a	2600 $\pm$ 113a	986 $\pm$ 168a	a
WW	PRLT2/89–33	29.95 $\pm$ 1.33b	36.20 $\pm$ 2.37a	1	1	1	1	1	5	5	5	5	3666 $\pm$ 287a	3238 $\pm$ 193a	a
WW	H77/833–2	38.45 $\pm$ 1.73a	39.31 $\pm$ 0.91a	1	1	1	1	1	5	5	5	5	3912 $\pm$ 195a	3756 $\pm$ 123a	a
WW	ICMR 01029	34.52 $\pm$ 1.71ab	39.23 $\pm$ 1.64a	1	1	1	1	1	5	5	5	5	3820 $\pm$ 240a	3680 $\pm$ 240a	a
WW	ICMR 02042	36.46 $\pm$ 1.59a	36.75 $\pm$ 1.51a	1	1	1	1	1	5	5	5	5	3614 $\pm$ 248a	3728 $\pm$ 175a	a

stay-green differences were consequences of variation in FTSW and NTR.

### Analysis of biochemical traits

#### Photosynthetic pigments

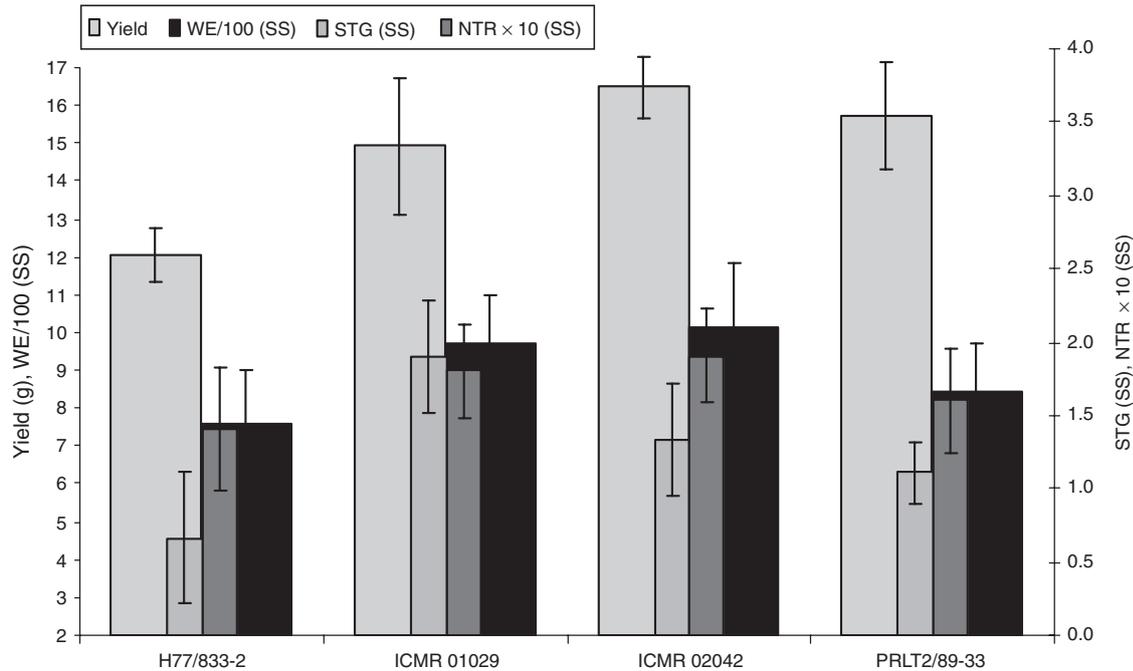
Drought decreased the photosynthetic pigment contents and ratios. The significant pigment degradation was observed under mild drought stress (except for Chl *b*) and under severe drought treatment for all pigments (data not shown). Chl *a/b* and Chl/Car ratio decreased significantly only under severe drought stress expressing the steeper decrease in Chl *b* than in Chl *a* and a more rapid degradation of Car compared with total chlorophylls content. Across treatments, tolerant genotype (PRLT2/89–33) contained less carotenoids than both QTL-NILs but neither QTL-NILs nor PRLT2/89–33 were different from sensitive H77/833–2. One-way ANOVA confirmed that NILs contained more carotenoids under WW. Under mild WS the tolerant genotype PRLT had higher Chl:Car ratio compared with ICMR2042, however, H77/833–2 and ICMR1029 didn't significantly differ from both of these genotypes (data not shown).

#### Anti-oxidative enzymes

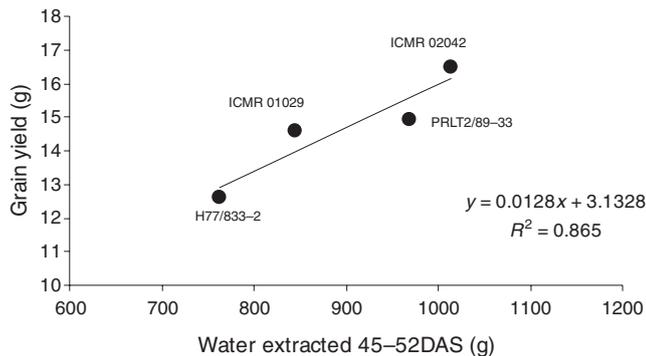
Eight SOD isoenzymatic forms were found (Fig. 1), i.e. four isoforms of MnSOD (band 1–4, MnSOD1–4), two isoforms of FeSOD (band 5–6, FeSOD1–2) and two isoforms of Cu/ZnSOD (band 7–8, Cu/ZnSOD1+2). Since the two (highly positively correlated) isoforms of Cu/ZnSOD were not distinguishable in the second sampling under SS, these two isoenzymes were summed and analysed together in both estimations. Most of enzyme activities across SOD isozyme spectrum did not change between drought stress samplings (Fig. 1; Table S1, available as Supplementary Material to this paper). Major isozyme Cu/Zn SOD1+2 showed an induction under SS treatment while minor isozymes, FeSOD2, showed an induction under MS treatment. QTL carrying genotypes did not differ. The block analyses across both samplings showed an induction of total SOD activity due to drought treatment.

Eight APX isoforms were separated during both sampling times (MS and SS) (Fig. 2; Table S1). There was a significant induction of APX2, APX4 under MS conditions and APX6 under both MS and SS. These trends proved significant for APX4 and APX6 using a block ANOVA across both samplings. Nevertheless, total APX activity didn't change significantly upon drought exposure. In both WW samplings tolerant PRLT2/89–33 had higher activities of APX 6 than H77/833–2 (sensitive genotype), whereas the activities of these isozymes were similar in NILs and H77/833–2. However, both WS treatments, especially SS, increased APX6 isozymes activities in NILs and PRLT2/89–33 (around 1 unit under SS) whereas APX6 of H77/833–2 slightly decreased. Similar variability was found in the case of APX 7. Across treatments, the APX7 activity of NILs and PRLT2/89–33 was higher than in sensitive parental genotype.

Two CAT isozymes (CAT1 and CAT2) were found under both treatments in both samplings (Fig. 3; Table S1). During the first treatment CAT1 isozyme accounted for around 19% of total CAT activity, in second sampling only around 8% of total CAT activity. Drought stress did significantly enhance the heavier



**Fig. 2.** Visualisation of the grain yield along with the parameters expressing soil-plant water status (water extracted (WE), stay-green score (STG), normalised transpiration (NTR)) from Table 1 in the severe stress (SS; 59 days after sowing) stage of terminal drought progression in pearl millet genotypes; PRLT2/89–33 (tolerant parent), H77/833–2 (sensitive parent), ICMR 01029 and ICMR 02042 (near isogenic lines). As a measure of variability (s.e.) bar is presented with each genotypic mean value. For better readability of the values distribution WE is divided by 100 and NTR multiplied by 10.



**Fig. 3.** Visualisation of the relation between grain yield and water extraction from Table 2 in later stage of terminal drought progression (45–52 days after sowing) in pearl millet genotypes; PRLT2/89–33 (tolerant parent), H77/833–2 (sensitive parent), ICMR 01029 and ICMR 02042 (near isogenic lines).

CAT1 isozyme activity in MS but the total CAT activity was not significantly affected.

*Correlation analysis*

*Correlation between physiological parameters*

There were tight positive relations between STG score, NTR, FTSW and WE (Table 2). We noted that these four traits tended to relate negatively to biomass but positively to yield under WS (see Fig. 3 for correlation to WE). Due to less points available for correlation we were able to prove only essential relations;

Significant negative relationship were found between NTR and stover and positive relation between STG ( $P < 0.01$ ), WE ( $P < 0.1$ ) and yield under WS (Table 2; Fig. 3).

*Correlation between photosynthetic pigments*

There was no significant relationship between means of Chl *a*, Chl *b*, Car and pigments ratios in WW plants, except a negative relation between Chl *a/b* and Chl *b* and Car. However, under drought all relationships between chlorophylls, carotenoids and their ratios were highly significant and positive (Table S2).

*Correlation between isoenzymatic activities*

In many cases, isoforms of same enzymes and often of different enzymes correlated significantly across both water regimes, although the water treatment affected the level of significance in some cases (Table S2).

*Correlation between photosynthetic pigments and enzymatic activities*

Generally, the activities of isoenzymes were tightly linked (positively or negatively) to the amount of pigments and their ratios – especially to the amount of Chl *b* and to the Chl *a : b* ratio. Usually, water treatment didn't affect the sign of the relation (positive/negative); However, in few cases (e.g. APX6, MnSOD3, FeSOD2, Cu/ZnSOD1+2) the water stress caused significant inversions in the relations to photosynthetic parameters; Table S2). We noted that as in the case of photosynthetic pigments the water stress tightened the

**Table 2. Correlation analysis between biochemical and agronomical characteristics separately in well-watered (WW) and water-stress (WS) conditions**

The observations from both sampling times (mild stress and severe stress, WW1 and WW2) were analysed together and the relation was described by Pearson correlation coefficients (r; i.e. numbers in the table). Relations highlighted with bold font stands for the significant relations at the level of x; (0.05 < P < 0.01), xx; (= < 0.01 < P < 0.001), xxx; (P < 0.001) and \*; (P < 0.1); correlation coefficients without × or \* sign are not statistically significant. NTR, normalised transpiration ratio; FTSW, fraction of transpirable soil water; STG, stay-green score; WE, water extracted between 45–52 days after sowing

Treatment	Trait	Chl a	Chl b	Chl a/b	Chl/Car	MnSOD1	MnSOD2	MnSOD3	MnSOD4	FeSOD1	FeSOD2	Cu/ZnSOD1+2	CAT1	CAT2
WW	Stover	-0.17	-0.23	-0.31	-0.07	-0.07	0.07	-0.21	-0.17	0.5	0.12	-0.21	0.12	0.01
WS	Stover	-0.06	-0.03	-0.19	0.04	0.16	0.42	0.04	-0.38	0.15	-0.01	0.2	0.01	-0.07
WW	Yield	-0.24	0.53	-0.55	<b>xx0.9</b>	-0.2	0.28	-0.05	0.58	-0.3	-0.41	<b>x0.82</b>	-0.79	<b>xx0.91</b>
WS	Yield	0.63	0.49	0.06	-0.18	0.31	-0.46	0.68	0.67	0.46	0.28	<b>x-0.80</b>	-0.18	0.24
WS	STG	<b>xx0.86</b>	<b>x0.82</b>	<b>xx0.85</b>	<b>xx0.84</b>	-0.62	<b>xx-0.84</b>	<b>x0.79</b>	-0.33	0.48	<b>x-0.81</b>	<b>xx-0.86</b>	<b>x0.71</b>	<b>x-0.75</b>
WS	NTR	<b>xx0.91</b>	<b>xx0.85</b>	<b>xx0.92</b>	<b>xx0.90</b>	<b>x-0.79</b>	<b>x-0.82</b>	<b>xx0.87</b>	-0.41	0.58	<b>xx-0.85</b>	<b>xx-0.89</b>	<b>xx0.90</b>	<b>xxx-0.93</b>
WS	FTSW	<b>xx0.92</b>	<b>xx0.88</b>	<b>xx0.9</b>	<b>xx0.89</b>	<b>x-0.77</b>	<b>x-0.80</b>	<b>xx0.85</b>	-0.45	0.46	<b>xx-0.90</b>	<b>xx-0.89</b>	<b>xx0.87</b>	<b>xx-0.88</b>
WS	WE	<b>xxx0.94</b>	<b>xx0.88</b>	<b>xxx0.95</b>	<b>xxx0.93</b>	<b>x-0.81</b>	<b>x-0.82</b>	<b>xx0.84</b>	-0.55	0.55	<b>xx-0.90</b>	<b>xx-0.85</b>	<b>xx0.87</b>	<b>xx-0.90</b>
Treatment	Trait	APX1	APX2	APX3	APX4	APX5	APX6	APX8	Stover	Yield	STG	NTR	FTSW	
WW	Stover	0.17	-0.12	-0.07	0.02	0.31	-0.07	-0.26	-0.26					
WS	Stover	-0.39	-0.27	-0.64	-0.4	0.38	-0.21	-0.36	-0.43					
WW	Yield	<b>x-0.85</b>	-0.75	0.69	0.02	-0.78	0.29	0.01	0.28					
WS	Yield	0.27	0.41	0.57	-0.51	-0.61	0.58	0.24	-0.38					
WS	STG	0.4	0.27	-0.19	-0.065	<b>xxx-0.93</b>	-0.66	0.18	-0.27	<b>xx0.93</b>				
WS	NTR	0.46	0.08	-0.22	-0.17	<b>xxx-0.93</b>	<b>x-0.77</b>	0.41	-0.5	0.62	<b>xx0.90</b>			
WS	FTSW	0.48	0.21	-0.26	-0.18	<b>xx-0.92</b>	<b>x-0.82</b>	0.3	<b>xx-0.93</b>	0.42	<b>xxx0.94</b>	<b>xxx0.98</b>		
WS	WE	0.34	0.21	-0.35	-0.11	<b>xx-0.90</b>	<b>xx-0.84</b>	0.3	-0.13	<b>0.83*</b>	<b>xx0.92</b>	<b>xxx0.97</b>	<b>xxx0.98</b>	

relationships between several isozymes and photosynthetic parameters (FeSOD2, APX5, APX6; Table S2).

*Correlation between biochemical and physiological parameters*

Under WS conditions, a majority of the significant relationships occurred between biochemical parameters and traits expressing water status of plant/soil under WS treatment (WE, FTSW, NTR, and STG). All photosynthetic pigment characteristics were positively related to WE, STG score, NTR and FTSW. Then, a majority of isoenzymatic activities were also related to WE, STG, NTR and FTSW; however, some of them positively and others negatively. Strong positive correlation to these three physiological parameters were identified with MnSOD3, CAT1 while FeSOD2, Cu/ZnSOD1+2, APX5, APX6 and CAT2 shared negative relation to WE, STG, NTR and FTSW. Few characteristics were related to grain yield under WW (Chl : Car, Cu/ZnSOD1+2, APX1, CAT1). But except Cu/ZnSOD1+2, which was negatively related to yield under WS (though positively under WW), none of the enzyme had any significant relationship to yield under water stress (Table 2). The biomass production was not significantly correlated with any of biochemical parameters (Table 2).

**Discussion**

Side-by-side assessment of biochemical, physiological and agronomical traits was made in several pearl millet genotypes contrasting for their terminal drought tolerance, with the objective of comparing the relative importance of biochemical and physiological traits on the resulting yield under terminal water stress. Results showed that although biochemical traits were clearly related to physiological traits reflecting plant and soil water status, none of the biochemical traits except one was related to grain yield under stress. In contrast, grain yield under stress was significantly related to water extracted during grain filling, and this led to differences in the plant and soil water status, including differences in the stay-green score. This showed that drought tolerance depended first on water status aspects and that the biochemical acclimation traits, here anti-oxidant enzyme activities, were not the primary drivers of yield differences under water stress, but were likely consequences on differences in the water status. Further, it shows that relevant comparison of biochemical indicator should not be made on a chronological basis, but rather on a plant water status basis.

*Comparison of physiological traits*

Water extracted was slightly higher in drought tolerant genotypes, although not significantly (P < 0.1) in the latest stage of drought (52–59DAS), but this led to significant differences in grain yield (Fig. 3). These minute differences in WE, which we interpret as consequences of water saving traits (Kholová *et al.* 2010a, 2010b), translated in significant variation in the soil moisture (FTSW) between genotypes under WS with sensitive parent H77/833–2 having the driest soil available for grain filling. The maximum differences in FTSW between genotypes were around 6% (approximately 0.6 L and total water extracted under stress around 12 L). These small nuances in soil moisture most probably resulted in genotypic differences in plants transpiration

relative to the well-watered control, with sensitive parent H77/833–2 attaining the lowest NTR. The relatively small variation in FTSW and NTR then led to apparent, visually observable, genotypic differences in a stay-green phenotype (STG) and that especially in MS where tolerant genotype PRLT2/89–33 and both NILs appeared greener (higher STG) than the sensitive parent H77/833–2. Consequently, these four highly correlated traits; water extraction (WE) *cum* soil moisture (FTSW) *cum* relative transpiration (NTR) *cum* stay-green scores (STG) under water stress showed similar genotypic variation as yield and were also correlated with yield under water stress. WE, FTSW, NTR and STG also tended to relate negatively to stover under WS though this correlation was significant only between FTSW and stover. These genotypic variations in grain yield and stover obtained were consistent with previous field (Serraj *et al.* 2005) and lysimetric observations (V. Vadez, J. Kholová, R. S. Yadav and C. T. Hash, unpubl. data). Sensitive parent H77/833–2 had significantly lower grain yield but higher stover yield under conditions of terminal drought compared with tolerant parent PRLT2/89–33 with both NILs having higher yield than the sensitive parent H77/833–2 (at  $P < 0.1$  for ICMR01029). Under well-watered conditions, the sensitive genotype and both NILs had similar or higher yield than tolerant parent. The inverse relation between biomass and yield under WS and its consequent link to traits expressing soil/plant water status may, in fact, add to our hypothesis that the constitutive genotypic differences (here the plant growth habits – biomass accumulation) can influence the water utilisation patterns (smaller plants would exhaust available water source slowly and would also have more water left for grain filling) leading to higher yield under these stress conditions (e.g. Hammer *et al.* 2006; Zaman-Allah *et al.* 2011; Belko *et al.* 2012).

### Comparison of biochemical traits

#### Photosynthetic pigments

Upon exposure to drought, the content of photosynthetic pigments significantly dropped at both investigated stages of drought exposure which is a typical symptom of oxidative stress (Smirnoff 1993). Nevertheless, photosynthetic pigments ratios (Chl *a/b* and Chl/*car*) significantly declined only under SS treatment suggesting that the Chl *b* and carotenoids contents under severe drought were relatively smaller compared with Chl *a* and total Chl respectively. Interestingly, the amounts of photosynthetic pigments contents were strongly associated with each other under water stress but much less under well-watered conditions. Such observations may mean that pigments might be in excess of photosynthetic needs under non-stress conditions. In any case, after the stress imposition in these field-like conditions, the measured photosynthetic pigments parameters did not clearly discriminate genotypes based on their genetic make-up (i.e. presence/absence DT-QTL), in agreement with our previous results (Kholová *et al.* 2011).

#### Anti-oxidative enzymes

With regards to SOD, the link between enhanced SOD activity and level of drought tolerance is still controversial (e.g. Quartacci and Navari-Izzo 1992; Badiani *et al.* 1993; Türkan *et al.* 2005;

Torres-Franklin *et al.* 2007; Simova-Stoilova *et al.* 2008; Kholová *et al.* 2011). Only the activity of Cu/Zn SOD1+2 and FeSOD2 increased under severe and mild drought, respectively. Similarly, induction of only Cu/ZnSOD isoform by drought was shown in wheat (Simova-Stoilova *et al.* 2008) and these authors couldn't discriminate between drought tolerant/sensitive wheat genotypes based on any changes in SOD isozymatic spectrum. This also agrees with our previous work, where variation in the SOD isoenzymatic spectrum could not discriminate tolerant and sensitive genotypes (Kholová *et al.* 2011).

Analysis of the APX isozymatic spectrum confirmed the results of a previous study (Kholová *et al.* 2011), although the pattern of minor APX isozymes detected slightly differed. Two minor (APX2 and APX4) and one major isozyme (APX6) increased its activity under drought, whereas total APX activity did not significantly increase. Such results agree with other report on pea (Mittler and Zilinskas 1994) or pearl millet (Reddy *et al.* 2009), where only certain APX isoforms proved the plasticity upon stimulation by particular stress type. This also indicates that methods based on total enzyme's activity might be misleading (similarly in Cruz de Carvalho 2008). Two major APX6 and APX7 activities appear to reflect presence/absence of DT-QTL within water regimes (these corresponded to APX5 and APX6 in Kholová *et al.* 2011). However, contrary to our previous study, these differences were clearly observable also under WW conditions, pointing out their rather constitutive character. Under WW conditions, the activity of APX6 was highest in tolerant parent (PRLT2/89–33). Both intensities of drought increased activity of these isozymes (APX6) in PRLT2/89–33 and both NILs, but only marginally in sensitive H77/833–2. APX7 activity showed no plasticity under water limitations. Therefore, we could discriminate between DT-QTL carrying materials from sensitive parental genotype (H77/833–2) based on APX6 and APX7 activities, in agreement with our previous results (Kholová *et al.* 2011).

Reports on CAT activity under drought stress are equally heterogeneous. CAT activity has been shown to increase (e.g. Rubio *et al.* 2002; Luna *et al.* 2004), remain unchanged, or decline upon drought exposure (Zhang and Kirkham 1994; Fu and Huang 2001; Türkan *et al.* 2005; Simova-Stoilova *et al.* 2008). Here, we confirmed the presence of two CAT isozymes found earlier (Kholová *et al.* 2011). However, in contrast to our previous work, the minor CAT1 isozyme was strongly active in well watered conditions, which may indicate it is also responsive to other environmental stimuli (e.g. high temperature, low relative humidity; high VPD). CAT1 activity was significantly induced at the earlier stage of drought stress. Induction of particular CAT isozyme was also found in work by Srivalli *et al.* (2003). However, such variation didn't change total CAT activity under drought treatment significantly.

#### Relation of physiological and biochemical traits

From the present study it was clear that all photosynthetic pigment characteristics were positively related to the water extraction (WE), fraction of transpirable soil water (FTSW), normalised transpiration (NTR) and stay-green score (STG); i.e. the traits expressing plant/soil water status. This can be expected since more water available at mild and severe stress would simply

delay the process of pigment degradation. This is an important finding because samples were collected at the same time, but soil and plant water status varied at these sampling dates. Therefore, this work suggests that future work on comparing the biochemical acclimation response to stress should not be made on a chronological basis but on a water status basis. It is probable that earlier studies showing differences in pigment characteristics could have simply been the consequences of differences in plant water status of the sampled plants. Unfortunately, traits related to plant water status were often not measured. In any case, none of these pigments characteristics was significantly related to yield under WS conditions.

The anti-oxidative isozymes followed the same trend. About half of them were strongly related, negatively or positively, to WE, FTSW, NTR and STG. We interpret that plant/soil water status was consequently reflected either in decreased (isozymes negatively related to WE, FTSW, NTR and STG) or enhanced (those positively related to WE, FTSW, NTR and STG) specific isoenzymatic activities. However, none of the isozymes was significantly related to seed yield under WS conditions, except Cu/ZnSOD1+2 which related negatively to yield under WS (this isozyme had also a strong positive correlation with yield under WW;  $R^2 = 0.82$ ). If any hypothetical link between these isozymes and yield could be induced, based on results presented we could speculate the basal activity of Cu/ZnSOD1+2 in WW might relate to the growth habits of the genotypes (recent evidences in work by Rizhsky *et al.* 2002; Pnueli *et al.* 2003; Im *et al.* 2009; Boss *et al.* 2010) and consequently might have turned into a penalty under WS. These results, therefore, indicate that there is likely no causal relationship between high/low activity of these anti-oxidative enzymes and the yield performance under terminal drought, but rather a consequential relationship that reflects differences in water extraction (WE), soil water content (FTSW) leading to higher transpiration (NTR) and maintenance of cellular activities ('stay-green' phenotype). Indeed, results presented appear to support our hypothesis that, at least in these lines, traits allowing water conservation expressed under well-watered conditions at vegetative stage (Kholová *et al.* 2010a, 2010b, 2012) would contribute to making more water available for the grain filling part (V. Vadez, J. Kholová, R. S. Yadav and C. T. Hash, unpubl. data), as it happened here. This agrees with our previous results (Kholová *et al.* 2011) and shows results including yield data collected under field-like terminal drought conditions. We would then argue that at least some of the previous work reporting a link between anti-oxidative enzymes and 'drought tolerance' are likely consequences of the same nature: differences in plant/soil water status explaining genotypic differences in tolerance and entailing differences in anti-oxidative enzymes' activities. We can still speculate that some of the tissue drought acclimation processes may have a role to play in some plant species and under some specific conditions (Tardieu 2011), but their positive effect on yield under severe terminal drought (i.e. restricted water availability during the season) in pearl millet cannot, at minimum, equal the positive effect of water conservation mechanisms.

## Conclusion

Higher yield under terminal drought tolerance was directly associated to the traits linked to plant/soil water status (WE,

FTSW, NTR, STG) whereas the yield-biomass association with a majority of investigated biochemical traits (photosynthetic pigments, enzymatic activities) appeared to be rather a secondary effect of genotypic differences in plant/soil water availability. There were two APX isoforms (APX6 and 7) whose variation in both water regimes (WW, WS) reflected the presence/absence of DT-QTL in investigated genotypes. Of these, only APX6, which was also identified in a previous study, responded to drought imposition. We interpret the relation of these APX6 and 7 to yield is not causal, but its persistent character, if functional, may rather point out to constitutive differences under well-watered conditions.

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