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Quantitative Trait Loci (QTL) for water use and crop production traits collocate with major QTL for tolerance to water deficit in a fine mapping population of pearl millet (*Pennisetum glaucum* L. R. Br.)

Murugesan Tharanya[1,2]. Jana Kholova[1]. Kaliamoorthy Sivasakthi[1,2]. Deepmala Seghal[3]. Charles Tom Hash[4]. Basker Raj[1]. Rekha Baddam[1]. Thiyagarajan Thirunalasundari[2]. Rattan Yadav[5]. Vincent Vadez[1*]

[1]International Crops Research Institute for the Semi–Arid Tropics (ICRISAT), Crop Physiology Laboratory, Patancheru 502324, Telangana, India.

[2]Bharathidasan University, Tiruchirappalli 620 024, Tamilnadu, India.

[3]International Center for Maize and Wheat Improvement (ICRISAT), Km. 45, Carretera Mex-Veracruz, El Batan, Texcoco, CP 56237, Mexico[4]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), ICRISAT Sahelian Center, Pearl Millet Breeding, BP 1204, Niamey, Niger.

[5]Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, AberystwythSY23 3EB, UK

[*] To whom correspondence should be addressed. E mail v.vadez@cgiar.org

Abstract

Key message **Four genetic regions associated with water-use related and agronomic traits across different levels of plant organisation were identified within the previously reported region for terminal water deficit adaptation on linkage group 2. The linkages**

between traits were analyzed using QTL co-localization approach and principal component analysis.

Abstract To increase yield across a range of water stress regimes, we require a precise understanding of biological mechanisms that eventually contribute to it, and an approach to decipher that is to assess the degree of co-mapping of genetic regions responsible for traits putatively involved in water stress adaptation and genetic regions responsible for agronomic traits measured in the field. For that, a fine-mapping population of pearl millet, segregating for a previously identified quantitative trait locus (QTL) for adaptation to terminal water deficit on linkage group 2 (LG02), was tested across different experimental environments (pot culture, high-throughput phenotyping platform, lysimeters, and field). This population was phenotyped for traits at different levels of plant organization, ranging from water-use traits (transpiration rate, leaf area, plant organ dry weights, etc.) to crop production and agronomic traits (grain yield, tiller number, harvest index, etc.) The linkages between traits across the experimental systems were analyzed using QTL co-localization approach and principal component analysis (PCA). The functional relevance of the phenotyping systems was traced by PCA analysis. Furthermore, four regions within the LG02-QTL underlying substantial co-mapping of water-use related and agronomic traits were found. These regions, identified across the experimental systems, provided genetic evidence of the tight linkages between water-use traits phenotyped at lower level of plant organization and agronomic traits assessed in the field. It suggests that combining phenotypic data captured at different levels of plant organization can deepen our understanding of the biological mechanism underpinning complex traits, thereby benefiting both geneticists and breeders.

Key words: Water stress, GxE interactions, high-throughput phenotyping, vapor pressure deficit

Introduction

Pearl millet [*Pennisetum glaucum* (L.) R.Br.] is the sixth most important global cereal crop (Sehgal et al. 2012) and an important source of livelihood for subsistence of farming communities of semi-arid tropics (SAT). Pearl millet is one of the few multipurpose crop options suitable for the rain-fed agriculture on marginal lands of SAT. It can produce significantly under water deficit/salinity/heat stress compared to other crops (Mahalakshmi et al. 1987; Krishnamurthy et al. 2007, Gupta et al. 2015). Though pearl millet could adapt to harsh environments, water deficit during the crop growth reduces its yield significantly (Mahalakshmi et al. 1987 & Bidinger et al. 1987).

Pearl millet crop improvement programs, involving mapping of complex traits, generally aim to localise genomic regions responsible for water deficit adaptation based on yield performance in targeted environments. However, there is generally a lack of understanding of the mechanisms of crop adaptations leading to crop production improvement in a given environment, and their genetic relationships, and tools to assess these mechanisms greatly hamper progress in crop production improvement (Banziger and Cooper 2001). In the case of pearl millet's adaptation to water deficit stress, the systems used till date were the field assessments for differences in panicle harvest index (PNHI) and yield (Bidinger et al. 1987); lysimeters (Vadez et al. 2011) to assess the difference in profile of water-use, which was then shown to contribute to increased yield under terminal water deficit stress; LeasyScan to assess the differences in canopy development in a high-throughput manner (Vadez et al. 2015) and pot culture to assess the difference in transpiration response to VPD (Kholova et al. 2012). In this study, we evaluated different phenotyping approaches to capture these mechanisms accurately and effectively using a fine mapping population segregating for traits mentioned earlier, with an aim to understand the relationships between traits measured at

different levels of plant organization, and to progress in the understanding of water deficit adaptive mechanisms and their relationships..

Several mapping studies analyzing the genetic basis of water deficit adaptation in pearl millet exist. A number of quantitative trait loci (QTLs) for grain and stover yield under terminal water deficit conditions were identified (Yadav et al. 2002, 2003, 2004; Bidinger et al. 2007). Among these, a major QTL for yield under terminal water deficit has been identified (Yadav et al. 2002) on pearl millet linkage group 2 (LG02) in two independent RIL populations (H 77/833-23 x PRLT 2/89-33 and ICMB 841 x ICMB 863B; Bidinger et al. 2005; Serraj et al. 2005). An analysis of the same populations showed that several QTLs for drought adaptive mechanisms (related to plant water use; e.g. transpiration rate Tr; organ weights, leaf area and thickness) co-localized with an originally identified QTL for yield maintenance under drought on linkage group (LG) 02 (Kholova et al. 2012 and Kakkera et al. 2015). However, phenotyping for traits related to plant water use in a large mapping population in pearl millet is time consuming and laborious work. For instance, Kholova et al. (2012), used pot culture to phenotype the water-use related traits (transpiration response to VPD) manually, which involved measuring the leaf area of hundreds of plants destructively. In this study, canopy development/vigor were not taken into account as it requires high throughput techniques. Also the pot culture was not suitable for assessing the yield related components. Therefore, in this work we investigate, compare, and link phenotyping outputs across various phenotyping systems; i.e. pot culture (Kholova et al. 2012), LeasyScan (Vadez et al. 2015), Lysimeters (Vadez et al. 2011) and field (Bidinger et al. 2007).

Hence, the overall objective of this study was to i) assess the variation in transpiration efficiency (TE) using lysimeters ii) map QTLs for traits related to plant water use and crop production traits using various phenotyping platforms iii) assess the associations between plant water use components and plant production traits through QTL co-localization approach

iv) develop functional understanding of associations between investigated traits through PCA and v) propose a crop improvement strategy accordingly.

Materials and Methods

Plant material – fine mapping population (FMP)

A major drought tolerant QTL (DT-QTL) for water deficit adaptation in pearl millet was identified earlier by Yadav et al. 2002. The introgression of this QTL into H77/833-2 (high yielding but poor water stress adapted) showed yield benefits across water-limited environments (Serraj et al. 2005). Phenotyping and mapping of traits underlying this DT-QTL has been shown to determine some of the water-use related parameters in the RIL population (Kholova et al. 2012). As the DT-QTL interval was large, a fine mapping population (high resolution cross) consisting of ~2500 individuals segregating specifically for DT-QTL interval on LG02 was established by crossing the best performing NILs of ICMR1029 with ICMR1004 (Seghal et al. 2012 and Yadav et al. 2010). This population was screened with 6 SSR markers (Xpsmp2237, Xpsmp2072, M13_Xpsmp2066, M13_Xpsmp3056, Xpsmp2206 and Xpsmp2059) and individuals were crossed with male sterile line 843A to avoid inbreeding depression (Yadav et al. 2010). Later 11 new SNP and CISP markers were added (Seghal et al. 2012) and therefore 17 polymorphic markers were used for mapping QTLs. 162 lines having all combinations of crossing-over between the markers were finally selected for the trials.

Plant growth conditions and phenotyping

In this work, the FMP segregating within DT-LG02 was tested using four different phenotyping environments to further elucidate the link between water use related traits and crop production parameters (Table 1- experimental overview). All experiments following were conducted at Patancheru – ICRISAT campus.

1) **Pot culture -** This experiment was done in a similar way as described in Kholova et al. 2012. Here the lines were evaluated in well-watered conditions for traits linked to water use (transpiration, transpiration rate, leaf area, root weight, leaf weight, specific leaf area, shoot weight; refer table 1) during February 2010 in outdoor conditions. The average day/night vapor pressure deficit (VPD) during plant growth was 3kPa /0.90kPa with 32/24°C and relative humidity 37/70°. Four replications were maintained and the sowing of each replication was done every 3-4 days sequence for logistical reasons (see below). Sowing was done in 20cm diameter pots, using 4hills/pot and 3-5 seeds per hill. After a week of sowing, plants were thinned to one plant per hill and two weeks after sowing, final thinning of 2 plants per pot were done. At the end of thinning, Di - ammonium phosphate (300mg/kg of soil) and urea (200mgkg^{-1} of soil) were added. Pots were weighed 3 times at 7:10 am., 10:10 am and 2:10 pm to measure the transpiration (ghr^{-1}). The pots were weighed following the same sequence so that the time between the pot weighing was identical for all pots. These timings were chosen to assess the transpiration so that the measurements were done respectively in a period of low and high evaporative demand. The average low VPD was 1.87kPa (between 7:10 am to 10:10) and the average high VPD was 3.56kPa between 10:10 am and 2:10 pm). After the 3rd weighing, pots were re-watered to pot capacity and the same procedure was repeated on the following day with the same set of plants. After the last weighing on the 2nd day, the plants were harvested and the leaf area (LA) was measured immediately using leaf area meter (LI3000 model, Li-Cor, Lincoln, Nebraska, US). The leaf area measured was used to normalise the transpiration to calculate the transpiration rate (gcm^{-}

143 $^{2}hr^{-1}$). Other parameters like leaf dry weight (LDW), root dry weight (RDW), stem dry weight
144 (StDW), shoot dry weight (ShDW = LDW+StDW), total dry weight
145 (TOTDW=ShDW+RDW) and specific leaf area (SLA = LA/LDW) were also measured.

146 **2) High throughput phenotyping platform - LeasyScan (LS) -** LS is an automated high
147 throughput phenotyping facility capturing the traits related to the plant canopy development
148 (for details see Vadez et al. 2015; www.gems.icrisat.org). The protocol for data extraction
149 (canopy size - 3dimensional (3D) & projected LA) and plant height) and the way for filtering
150 data were described in Vadez et al., 2015.

151 Here the plants were grown under well-watered conditions in two experiments carried out in
152 May 2015 and February 2016 and traits linked to canopy conductance (evapotranspiration,
153 transpiration and transpiration rate) and growth related traits (3D leaf area (leaf area from 3D
154 image captured by the scanner), projected leaf area (unshaded leaf area), canopy structure,
155 biomass production, tiller count) were collected (Table 1). Each replication/sector consisted
156 of two pots (20 cm diameter each) and each pot had 2 plants after final thinning, in a sector
157 area of 40x65 cm, i.e. approximately a quarter square meter. Pot filling was done with 12kg
158 Alfisol collected from the ICRISAT farm. Four hills per pot were made and 3-4 seeds were
159 sown per hill. First thinning (1plant/hill) was done at 8 days after sowing (DAS) and final
160 thinning was done at 14DAS so that 2 plants per pot were maintained. At the end of final
161 thinning, plant count was done to record the number of plants per pot. Watering was done
162 either early in the morning or late in the afternoon. Top dressing was done with Di-
163 ammonium phosphate (300mg/kg of soil). The data from LeasyScan were collected through
164 either automated through scanning machine or gravimetric methods.

165 The scanning of the canopy started after the last thinning and the scanned data on leaf area
166 (3D &projected LA) and plant height were recorded for every 2 hours. Data visualisation and

extraction were done through Hortcontrol (Vadez et al. 2015). A gravimetric assessment of plant transpiration in this setup, similar to the one above, consisted of weighing pots on the 4th week of plant growth and weighing were done in both years. Pots were watered on the day before weighing to bring them to field capacity. Weighing was done in the morning (8:00-10:00 am) and the afternoon (3:00-5:00p.m) to measure evapotranspiration. Empty pots at field capacity (5 reps) were also weighed to estimate the soil evaporation. Soil evaporation was estimated from the leaf area index, so that the transpiration (T; g) value of each sector could be calculated from the evapotranspiration (ET; g). The estimation consisted in considering that soil evaporation (E_S) would be close to zero at a leaf area index (LAI) of 2, and would be equal to the evaporation of a bare soil (E_{BS}) at a LAI of 0. Therefore, the soil evaporation of each sector (E_S) was proportional to the LAI so that:

$$E_S = (1-LAI/2)*E_{BS}$$

By dividing ET and T with 3D-LA, evapotranspiration rate (ETr; $gcm^{-2}hr^{-1}$) and transpiration rate (Tr; $gcm^{-2}hr^{-1}$) were calculated. Projected leaf area growth rate (PGR; cm^2day^{-1}) and 3D-LA growth rate (3DGR; $cm^2\ day^{-1}$) were calculated based on the average differences in respective leaf area between consecutive days of exponential growth phase. The scanners measured both the 3D-LA and the projected LA (PLA) and both parameters are closely related. However, the PLA representing the vertical projection of the 3D-LA on the ground, there is a degree of difference between these two indices that reflect somewhat the angular position of the leaves in the canopy. Therefore, PLA was regressed against the 3DLA and the residuals from the linear relationship between PLA and 3DLA were calculated as the difference between the observed PLA and the predicted PLA from the regression equation. For the sake of simplicity, these residuals were referred to as canopy structure (CS). Other parameters like shoot dry weight (ShDW; g), Tiller numbers (TNO), specific leaf area (SLA;

191 gcm^{-2}) and specific leaf weight (SLW; cm^{-2}) were also recorded and computed after harvest
192 and drying of the plant samples.

193 **3) Lysimeter -** For experiment 3, protocol for growing and testing plants in lysimeters were
194 followed according to Vadez et al 2013. The lysimeters offer an experimental setup that helps
195 in assessing both water-use and crop production traits over the entire cropping cycle. Four
196 hills per PVC cylinder were sown on February 13th, 2010 and the experiment lasted till April
197 29th 2010. The average day/night temperature during plant growth was 36/20°C and relative
198 humidity 30/75°C. Two weeks after sowing the seedlings were thinned to 2 per cylinder and
199 finally thinned to one per cylinder after 3rd week of sowing. Urea was applied as to dressing
200 (1.38gN/plant) at 28 DAS. Full irrigation was given until 28 days after sowing (DAS). Each
201 cylinder received 500ml of water twice a week until 14 DAS and 500ml of water on alternate
202 days until 28DAS. At 28DAS, the soil in the cylinders was covered with polythene beads to
203 prevent direct evaporation. Weighing were done at 36, 41, 50, 57, 64DAS. The average
204 day/night temperature during plant growth was 36/20°C and relative humidity 30/75°C. The
205 plants were tested under gradual water deficit conditions in the way that irrigation was
206 stopped at panicle emergence stage. The parameters bridging the water use and crop
207 production were assessed. Transpiration was calculated based on the differences in cylinder
208 weights and added water. Phenotyping of stay green (STG) was done by visual scoring at
209 60DAS. At 76DAS plants were harvested and the main tillers and secondary tillers were
210 separated. After drying in hot air oven at 70^{o}C for 3days, organ dry weights like main plant
211 shoot dry weight, tiller shoot dry weight, main plant panicle dry weight, total panicle dry
212 weight and the total biomass were recorded. Weight of grains per plant (including tiller
213 grain), tiller grain yield, 100 grain weight, number of tillers, main plant panicle dry weight,
214 total panicle dry weight (main plant panicle and tiller panicle; refer table 1). The panicle
215 harvest index (PNHI) was calculated as the ratio of grain weight to the total panicle weight.

Transpiration efficiency (TE) was calculated according to Vadez et al 2013, by dividing the total biomass produced (panicle and vegetative tissues) by the total transpiration post anthesis (36-64DAS). Here the biomass prior to initiation of transpiration assessment was not measured and then was not deducted from the TE assessment. Here it was assumed that this initial biomass was small compared to the final biomass, and was similar across all lines, so that its influence on the overall TE value would be small and the effect on the genotypic differences even smaller.

4) Field - For Experiment 4, standard field management practices for millet cultivation were followed (Bidinger et al. 1987). The crop was raised during the summer rain-free season (January to April of 2010 & 2011) with 4 replicated plots (2 rows of 4 m) in an α-lattice design with randomized blocks within each treatment. Three types of water stress (treatment were followed –Well- watered, mild water stress and severe water stress. The severe water stress treatment was imposed at the time of booting by cessation of watering. The mild water stress treatment differed from severe water stress by receiving one additional round of irrigation (50mm) in comparison with early stress on the following week after the irrigation was stopped in severe water stress treatment. The well watered (control) received water until grain filling. This experiment was focused on the evaluation of crop production parameters (tiller numbers (TNO), grain yield (GY) thousand grain weight (ThGW), grain number per panicle (GNP^{-1}), tiller panicle dry weight (TPNDW), tiller grain weight (TGW), harvest index (HI), Panicle harvest index (PNHI), time to flowering (TF), panicle diameter (PD) and panicle length (PL). Grain yield was calculated as kg of grain obtained per plant. Harvest index (HI) Panicle harvest index (PNHI) was calculated similarly as in the lysimeters. Time to flowering (TF) is calculated as number of days taken to attain the flowering stage. Panicle length (PL) and panicle diameter (PD) were measured (in cm) after harvest. Tiller number (TNO) was recorded as the number of tillers (includes all, either panicle producing or non-

panicle producing) produced per plant. Stover dry matter yield (SDMY) was calculated as kg of stover obtained per plant. Total dry weight (TOTDW) was calculated as the sum of stover dry matter yield (kg) per plant and panicle yield per plant. In this experiment, only 144 genotypes were tested unlike other above experiments where 162 genotypes were tested. Grain number/panicle ($GNPN^{-1}$) was calculated as number of grains produced per panicle. Thousand grain weight was calculated as weight (g) of thousand grains (3 replications) dried in oven for 3 days at $70^{\circ}C$.

Data analysis & statistics

ANOVA (GenSTAT version 12) was employed to evaluate the range of variation for the traits within the experiments. Simple correlation (crop production related traits from field) and principal component analysis (name of the package princomp or some other?? executed in R software) for the traits across different experimental environments were done to evaluate the relations among them. Firstly, the relationships between the traits from the field environment were analysed within the specific water stress treatment (well- watered (WW) and severe water stress (SS)). Then to visualise the relationship between GY from field (both years) towards the traits from other environments, GY (SS) was compared to traits measured in the lysimeters (SS) and pot culture (WW) experiments. GY (WW) was compared with traits measured in the LeasyScan (WW) experiment. In addition, an attempt was made to test possible relationships between early water extraction (T36DAS and T41DAS in the lysimeters (i.e. prior to water stress onset) and canopy development traits assessed in the LeasyScan platform (3DLA, PLA, 3DGR, PGR, CS and PH).

Finally, the composite interval mapping (CIM) study was used to evaluate and visualize the quantitative trait loci (QTLs) and their effect within the population using QTL cartographer (WinQTL 2.5). The experimental design opted for this mapping study was selfed intercross

line (SF) and map function used was Haldane. BLUPs mean (GenSTAT version 12) were used for both PCA and composite interval mapping. Broad sense heritability was calculated using $h^2 = \sigma^2_G/(\sigma^2_G + \sigma^2_E)$ where σ^2_G is the genetic variance σ^2_E is error variance (Kholova et al. 2012) from GenSTAT (version 12).

Regarding the production traits from the field environment, there was high variation in the interaction of genotype with water stress treatment across the two different years (data not shown. Therefore the mapping of production traits from the field was done individually for each year and treatment. Similarly, the mapping of traits from LeasyScan were done individually for two different years.

Comment [SD(1]: may be here you can just give rank correlation to show g x e interactions

For the pot culture trial, the sowing of the four replications each with 162 entries were done in sequential manner with four days interval between the successive sowing of each replications due to logistical reasons as it involves the manual weighing of the many pots and destructive measurements of leaf area for water use related traits (Tr, LA). When the blups mean of all four replications were used for mapping purpose, none of the traits phenotyped using pot culture were mapped (data not shown). One of the possible reason could be the differential effect of VPD on the plant growth and water-use related traits (Kholova et al. 2015). On the other hand, when we used the individual replications for mapping purpose as in Kholova et al., 2012, we identified many QTLs for canopy development, water use and biomass related traits (see supplementary table 3 and 4). As this way of analysis resulted in too many QTLs which was quite different from than the other analysis that used blups mean (in case of LeasyScan, lysimeters and field trials), we did not use these QTLs mapped from individual replications (pot trial) to compare with the QTLs from blups mean (LeasyScan, lysimeters and field trial).

Result

Transpiration efficiency (TE) variation and its relationship between GY, HI and post anthesis water extraction

TE was assessed using lysimeters and it was significantly different among the genotypes under severe water stress (SS). It ranged from 3.43 to 4.50 gkg^{-1} with a mean value of 4.00 gkg^{-1}. Regression analyses were done among the grain yield (GY), harvest index (HI), TE and post anthesis water extraction (T36-64DAS; Fig 1). The relationship between GY and HI was highly significant (R^2=0.835; p<0.001). Since GY and HI are auto-correlative in nature (Vadez et al., 2016) as GY is the part of HI, the residual variations unexplained by HI were computed according to Vadez et al., 2007 as the difference between the observed yield values and yield values predicted by the regression equation. Residual yields were plotted against TE and water extraction during post anthesis (36-64DAS; Fig 1). There was a significant positive correlation between residual GY variations with TE (R^2=0.335; p<0.001) and water extraction (R^2=0.17; p<0.05).

Summary statistics

The list of traits measured at different level of plant organization at different phenotyping environments were classified and described according to their functionality and complexity: (i) canopy development traits (assessed in LeasyScan and pot culture), (ii) water use traits (assessed in LeasyScan, pot culture and lysimeters), (iii) biomass and components (assessed in LeasyScan, pot culture, lysimeters and field) and, (iv) agronomic traits (assessed in lysimeters and field).

A) Canopy development traits - The canopy development traits included both those measured non-destructively in the automated LeasyScan platform and those assessed

312 destructively in the pot experiments; i.e. 3D Leaf area (3DLA), projected leaf area (PLA), 3D
313 leaf area growth rate (3DGR), projected leaf area growth rate (PGR), canopy structure (CS),
314 plant height (PH), measured with LeasyScan, and destructive leaf area (LA) and specific leaf
315 area (SLA), measured in a pot culture experiment (details in Table: 1). The genotypic
316 variation for the canopy development traits was highly significant ($p<0.001$) with high
317 heritability (43-84%) for the year 2015 whereas in 2016 only few traits showed significant
318 variation (PLA and PH) with 59-61% heritability. The significant range of variation
319 ($p<0.001$) obtained for 3DLA with 71% heritability from LeasyScan in 2015 were shown in
320 Fig 2A. The destructive LA and SLA measured manually in the pot culture experiment did
321 not show significant variation and had very low heritability (<10%).

322 **B) Water use traits -** The water use traits were measured in LeasyScan (ET, ETr, T, Tr), pot
323 culture (TrM and TrE) and in lysimeters (T36DAS, T41DAS, T50DAS, T57DAS, T64DAS)
324 (see details in table 1). The traits measured through LeasyScan had significant genotypic
325 variation ($p<0.001$) for 2015 and 2016 except T in 2016. Transpiration rate in the morning
326 (TrM) measured from pot culture and transpiration (T64DAS; water extraction at later stage
327 of crop development) from lysimeters also showed significant genotypic variation ($p<0.001$
328 & $p<0.05$ respectively) with 32% and 27% heritability respectively. The significant range of
329 variation ($p<0.05$) obtained for T64DAS through Lysimeter were shown in Figure 2B.

330 **C) Biomass traits -** The biomass traits were measured through pot culture (LDW, StDW,
331 TOTDW, RDW, ShDW), LeasyScan (ShDW, SLW), Lysimeter (TOTShDW, MShDW,
332 TShDW) and field (TOTDW, SDMY) see details (table: 1). Among these, the traits from
333 LeasyScan (ShDW, SLW), Lysimeter (TShDW), field (TOTDW-2010 WW; 2011 SS,
334 SDMY -2010 WW and MS) showed significant ($p<0.05$) genotypic variation with moderate
335 heritability (20-29% ; Table 2). The significant range of variation ($p<0.05$) obtained for
336 TOTDW from field in 2010 with 25% heritability under WW were shown in Fig 2C.

D) Crop production traits - The production traits were measured from LeasyScan (TNO), lysimeters (TNO, ThGW, MPNDW, MGDW, TPNDW, GY, TGW, HI, and PNHI) and field (GY, PNHI, TF, PL, PD, TNO, HI, ThGW, and $GNPN^{-1}$) (see details table: 1). Most of the production traits showed significant genotypic variation ($p<0.001$) with different water stress treatment and years (table: 2). Among them, the traits from field i.e. ThGW in 2011 (88% heritability in WW and 85% in MS) and 2010 (87% heritability in MS) and TF in 2011 (MS; 87% heritability) had the highest heritability of all traits. The significant range of variation ($p<0.001$) obtained for GY from field in 2010 under MS with 37% heritability were shown in Fig 2D.

QTL mapping

QTL mapping revealed that most of the traits were associated with four main genetic regions within the fine mapped region in LG02. Therefore for simplicity, the genetic regions are further referred as - region1 (R1) covers from191-205cM, region2 (R2) covers from 229-233cM, region3 (R3) covers from 236-240cM and region4 (R4) covers from 251-259cM.

QTL mapping for canopy development traits - For PLA, one major QTL (LOD 3.7 & PVE 34%) was mapped at R4 in 2016 (Fig. 3). Similarly for PGR, one major QTL (LOD 7.1 & PVE 49%) was found in R4 (Table 3). For these two traits (PLA and PGR), no QTLs were identified in 2015. Two major QTLs for PH were mapped in both 2015 (LOD 3.4 & PVE 52%) and 2016 (LOD 3.8 & PVE 14%) in R4 and R3 respectively (Table 3). The residual from 3DLA and PLA (so-called canopy structure, CS) was mapped in both 2015 (LOD 10.8 & PVE 32%) and 2016 (LOD 3.1 & PVE 10%) in R1 (Table 3). The alleles for the canopy development traits were contributed by both ICMR1029 and ICMR1004 (Fig. 3).

QTL mapping for water-use related traits - For water use traits (ET, ETr, T, Tr, TrM, TrE, T36DAS, T41DAS, T50DAS, T57DAS, and T64DAS), a total of 11 QTLs (both major and

minor) were identified. Among these, 8 major QTLs explaining 10-47 % of phenotypic variation were mapped in R1 (5 QTLs), R3 (1 QTL) and R4 (2 QTLs) (Table 3). For these same traits, 3 minor QTLs explaining 2-9 % of phenotypic variation were identified in R2 & R4 (Supplementary table 1).

Mapping details of water use related traits in LeasyScan and lysimeters are provided in Table 3 and supplementary table 1. In the Lysimeter system, STG trait had one major QTL (LOD 3.3 & PVE 10%) in R3 (Table 3). In 2015, two major QTLs for T (LOD 3.4 and 4.9 and PVE 27-37%) were mapped in R1 and R4 (Table 3). T57DAS had one minor QTL (LOD 2.8 & PVE 6%) mapped in R2 (Supplementary table 1). For Tr, one major QTL explaining (LOD 2.8 and PVE 13%) was mapped in R1 and another one minor QTL (LOD 2.5 and PVE 2%) was mapped in R4 (Supplementary table 1). In both years for ET, two major QTLs (LOD 3& 9.5 & PVE 24& 32%) were mapped in R1 (Table 3). In 2016, two major QTLs (LOD 9.2 & 9.6; PVE 36 & 47% respectively) for ETr were mapped in R1 & R4. Another minor QTL for ETr in 2015 was mapped in R4 (LOD 3.8 and PVE 9%) (Supplementary table 1). Most of the positive alleles for the water-use related traits were inherited from ICMR1029 (Fig. 3).

QTL mapping for biomass related traits - For biomass traits (SLW, ShDW, TShDW, SDMY, TOTDW), 11 QTLs were found across different experimental systems. Among these, 6 major QTLs (LOD 2.6 -12.6 and PVE 11 - 55%) were mapped in R1, R2, R3 & R4 and remaining 5 minor QTLs (LOD 2.6 -4.6 and PVE 1 - 9 %) were mapped in R2, R3 & R4 (Table 3 & Supplementary table 1). For SLW, two major QTLs (LOD 4.7 & 12.6 & PVE 40 & 55%) were mapped in R1 and R4 (Table 3). For ShDW (main plant shoot+ tiller shoot dry weight) one major QTL (LOD 3.9 & PVE 22%) was mapped at R1 and another minor QTL (LOD 4.6 &PVE 1%) was mapped at R4. For TShDW (tiller shoot dry weight), one major QTL (LOD 3.9 & PVE 11%) was mapped at R2 and another minor QTL (LOD 3.45 & PVE 9%) was mapped at R3 (Table 3 & Supplementary table 1). For SDMY, one major QTL

(LOD 2.6 and PVE 11%) was mapped in R2 (Table 3). For TOTDW, four QTLs were identified. Among these, one major QTL (LOD 3.7 & PVE 27%) was mapped in R3 and remaining three minor QTLs (LOD 4.2 -2.6 & PVE 6-8%) were mapped in R2 and R3 (Table 3 & Supplementary table: 1). Most of the positive alleles for biomass related traits were contributed by ICMR1004 (Fig 3).

QTL mapping for crop production related traits - For grain production related traits 82 QTLs were identified in three different systems (LeasyScan, Lysimeters and field systems). Among these, 65 major QTLs (LOD 2.5 -23.6 and PVE 10-56%) were mapped in R1, R2, R3, R4 and also in the regions between R1 and R2; R3 and R4 (Table 3). Remaining 17 minor QTLs (LOD 2.6- 19 and PVE 0-9%) were mapped in R1, R2 & R3 and also in the regions between R1 and R2 (Supplementary table 1). For GY, four major QTLs (LOD 3.1-8.0 and PVE 10-43%) were identified all under SS in the regions of R2, R3 and R4. For HI, six major QTLs (LOD 2.8-23.6 and PVE 10-43%) mapped in R2, R3 & R4.

For PD two major QTLs (LOD 2.7& 3 and PVE 10&56%) were identified under in R4 and in the regions between R1 and R2. For PL, three major QTLs (LOD 2.5-3.5 and PVE 10-34%) were mapped under WW and MS in R1, R2& R4 and one minor QTL (LOD 2.8 and PVE 5%) under MS was found in R3 position. For PNHI, 10 QTLs were identified across MS and SS in field and lysimeters systems. Among these, eight major QTLs (LOD 3.4 -8 and PVE 11-43%) were mapped in R2, R3 and R4 (Table 3). The remaining two minor QTLs (LOD 2.8-2.9 and PVE 1-3%) were found in R1 and in the regions between R1 and R2 (supplementary table 1). For TF, 14 major QTLs (LOD 4.8-11.8 and PVE 22-37%) were mapped in the regions of R2, R3 and in the region between R3 and R4. For TGW, three major QTLs (LOD 3.3-10.8 and PVE 28-37%) were mapped in R2, R3 and R4. For the ThGW, 13 major QTLs (LOD 2.9 – 10 and PVE 10-31%) were mapped in the regions of R2, R3, and between R3 & R4 position. For TNO, seven major QTLs explaining (LOD 3.1-6.4

and PVE 12 to 17 %) were mapped in the region of R2, R3 and in the region between R1 and R2. One minor QTL (LOD 2.7 and PVE 9%) was also found in the region between R1and R2. For TOTPNDW three major QTLs (LOD 2.7 – 6.4 and PVE 20-40%) under SS were identified in R2, R3 and R4. For TPNDW, two major QTLs (LOD 3.5&6 and PVE 17 & 45%) were mapped in R3 and R4. For $GNPN^{-1}$, one minor QTL (LOD 2.6 and PVE 9%) under MS was mapped in R3 position (Supplementary table: 1). Simple correlation analysis showed that most of the parameters from the field were closely related under specific water stress treatment in both the years (Table 4 and 5).

QTL co-localisation

In the R1 region, most of the QTLs for traits related to canopy development, water-use, and few biomass and crop production traits (mostly under WW & MS in field) co-located. Similarly, in the R4 region, most of the QTLs for traits related to canopy development, water-use, biomass and few of the crop production traits (mostly under SS in field) co-located. In the R2 region, most of the QTLs for crop production traits collocated with selected biomass (TShDW and SDMY; Fig. 3). In the R3 region, most of the QTLs for crop production traits collocated with biomass and few canopy development (PH) and water use (STG) related traits. Interestingly, most of the positive alleles for the crop production related traits under SS were contributed by ICMR1029 though the alleles from both ICMR1029 and ICMR1004 contributed more or less equally under WW and MS (Fig: 3).

PCA analysis

The purpose of the analysis was to do a PCA for individual treatment in field i.e. WW (Fig. 4a) and SS (Fig 4b) and then link these to the rest of the trials to illustrate trait associations. Data on GY from field (SS) and traits from lysimeters (SS) were combined in Fig 4c; Data on GY from field (WW) and traits from high throughput phenotyping platform (WW) were

435 combined in Fig 4d; Data on GY from the field (SS) and traits from pot culture (WW) were
436 combined in Fig 4e; traits on early water extraction (T36DAS and T41DAS) from lysimeters
437 (SS) and canopy development related traits (3DLA, PLA, 3DGR, PGR, PH) from LeasyScan
438 (WW) were combined in Fig. 4f.

439 In the field environment, the first three components explained 62% (2010) and 66% (2011) of
440 the variability under WW, 66% (2010) and 72 % (2011) variability under MS and 71% (2010
441 and 2011) under SS. Under WW, increase in tiller numbers (TNO) favoured grain yield (GY;
442 Fig. 4a) whereas under SS there was no such relationship, both in the field (Fig. 4b) and in
443 the lysimeters (Fig: 4c). Under SS, when the GY from both the years were combined with the
444 traits from the lysimeters, GY increased with increase in water uptake at the late stage of
445 plant development i.e. transpiration at 50, 57 and 64 days after sowing (50DAS, 57DAS and
446 64DAS; Fig: 4c). Interestingly GY from field under SS (2011) increased with STG scored at
447 60DAS (Fig 4c) which in turn was very closely related late water extraction (T64DAS). This
448 also supported the finding described above as the QTLs for grain yield (MS) and T57DAS
449 (SS) collocated with each other. Also the QTLs for GY from lysimeters (SS) collocated with
450 the QTLs for STG. The TOTDW from lysimeters increased with increase in TE under SS
451 (Fig: 4c).

452 Under WW and MS, the QTLs for GY collocated with CS (Fig 3). When the GY from field
453 under WW was combined with LeasyScan traits (WW), GY from the 2011 field trial
454 increased with increases in canopy structure (CS) and GY from 2010 field increased with
455 increase in projected leaf area (PLA; Fig. 4d). It was interesting to note that CS had influence
456 on the crop production related traits in addition to the water use i.e. transpiration. When the
457 GY from field (WW) was combined with pot culture traits (WW), GY increased with
458 increase in root dry weight (RDW), specific leaf area (SLA) and transpiration from pot
459 culture in the evening, i.e. under high VPD (TrE; Fig: 4e). The relationship between early

460 water extraction (T36DAS and T41DAS from Lysimeter; SS) and canopy development
461 related traits (3DLA, PLA, 3DGR, PGR, CS and PH from LeasyScan; WW) showed that,
462 increase in water extraction at early stage favoured the increase in CS (Fig: 4f).

463

464 **Discussion**

465 In this study, we identified four regions within the LG02 (191-258cM) associated with water-
466 use related traits and agronomic traits. These four QTL regions encompassed variability in
467 traits across different levels of plant organisation and these were phenotyped at different
468 experimental systems (canopy development and water-use related traits; biomass and
469 agronomic-performance related). Their common genetic co-localization allowed us to
470 speculate on their functional association.

Comment [SD(2)]: I don't think that we should mention this total interval as it looks very large even after using a fine mapping population

471 **Main detected QTL regions**

472 Firstly, we were able to trace back the locations of QTLs for similar traits documented in
473 RILs population before (Yadav et al. 2002 & 2004, Bidinger et al. 2007, Kholova et al. 2012
474 and Kakkera et al. 2015) and these fell into the regions as documented here; i.e. traits from
475 canopy development (leaf area – (PLA in the case of present study)), water use (T, Tr),
476 biomass (ShDW, TOTDW) and crop production (TF, ThGW, SDMY, $GNPN^{-1}$, GY, PNHI,
477 details in supplementary table: 2).

478 The plant traits related to canopy development and water-use mapped mostly in R1, R3 and
479 R4 while plant traits related to biomass and grain production mapped mostly in R2, R3 and
480 R4 position (Fig.3). Therefore, regions R3 and R4 appeared to underlie variability in all traits
481 across the phenotyping systems while R1 and R2 ?? appeared to have a more specific role
482 during early and later plant development stages, respectively.

The co-localization of traits across the systems in R3&R4 loci was very clear right from the early plant development till the crop production stage and their possible functional linkage is explained below. On the contrary, R1 appears to be rather specific to traits variability measured during early plant development (e.g. canopy structure - CS determined by specifically R1) while R2 locus underlined traits during later plant development (e.g. TF, TNO and PNHI determined by specific R2 and common R3 and R4). This suggest that measured traits variability is the consequence of presence/absence of several different loci, and each of these could relate to different simpler biological processes. In the sections that follow, we attempt to interpret the mechanistic of complex traits co-localization using co-mapping approach and multi-factorial regression (PCA).

Effect of water extraction during grain filling under water stress

Under SS, in field, PCA showed that GY was positively associated with amount of water available for extraction during grain filling (lysimeters; T50, 57 and 64DAS) and also reflected in the stay-green scores (STG; lysimeters). The expression of these traits during later plant growth (i.e. the positive association between GY and the water extracted at different times during the grain filling, T50, 57, 64DAS, and then the expression of a stay green phenotype with the positive association to water extracted at grain filling) might have been pre-determined by the magnitude of saving water from early water extraction (lysimeters; T36 and 41DAS; Vadez et al. 2011b, 2013a, 2014, Zaman Allah et al. 2011a). Also the regression analysis showed that GY was related to post anthesis water extraction indicating the importance of water availability during grain filling. There are reports stating that the water extracted during grain filling led to increase in yield (Manschadi et al. 2006, Kirkegaard et al. 2007 and Vadez et al. 2013a). The relationship between GY and TE under SS became stronger when the part of GY variation explained by HI was removed. This reveals the importance of TE on GY under water limited environments that has been reported

earlier (Hammer et al. 1997, Sinclair et al. 2005, Xin et al. 2009 and Vadez et al. 2011b). These results also highlight the importance of lysimetric system that can be used to precisely assess the water use yet approximating the field conditions.

Effect of Canopy structure

In the paragraph above, we showed the importance of limited plant early water-use for making more water available post-anthesis and then boosting production under severe water stress. The early water use related traits (lysimeters) was found to be associated with CS (in LeasyScan; Fig.4f) in this particular fine-mapping population. A high CS value represent a high residual in the relationship between the 3D leaf area and the projected area, which can be taken as a proxy for the degree of erectness of the canopy. Our interpretation is that high CS would also contribute to less leaf shading and may result in more transpiration and vice versa. Also the QTL for CS (WW) was found to be collated with the QTLs for GY (Field; WW & MS) and TNO (LeasyScan; WW). Therefore, this result not only emphasizes the importance of canopy organisation in space for crop early water-use but also highlights its importance for GY as CS determines the intensity of light penetration (Sampson et al. 1993) and photosynthesis (Pendleton et al. 1968, Intrieri et al. 1997, Stewart et al. 2003, Hammer et al. 2008 and Sharma et al. 2013).

Effect of tillering and biomass on grain yield

As expected, in WW, the crop grain production was the consequence of plants ability to accumulate biomass and partition the stored assimilates into the grains (Liang et al. 2009). This was also supported by the QTL mapping where the QTL for TNO collocated with the QTL for GY from field under WW and MS between R1 & R2 and also with GY under SS from field in R3 and lysimeters in R2 and R3. This was also highlighted in the PCA as the

grain production (GY) under WW was very well related to TNO under WW which indicates that grain filling in tillers under WW would add to the GY from the main plant panicle.

Similarly the QTL for TOTDW under WW in R3 collated with TF (WW, MS and SS), TNO (WW & SS), ThGW (WW, MS & SS), PNHI ((MS & SS), HI (MS & SS), $GNPN^{-1}$ (MS), and with TGW, TPNDW, TOTPNDW, GY (SS). The PCA analysis shows that under WW, TOTDW and SDMY were related to $GNPN^{-1}$ and under SS, SDMY is related to TNO. In other words, this R3 QTL appeared to represent a QTL for biomass. Similarly Liang et al. 2009, Shi et al. 2009, Matsubara et al. 2016 reported that QTLs for biomass, GY and its related component traits were found to be co-localised. QTLs for biomass related traits within the pearl millet LG02 were reported earlier by Kholova et al. 2012 and Kakkera et al. 2015.

On the contrary, under SS, the plant yield was delimited by its capacity to extract the soil moisture during grain filling. This was apparent from QTL co-localization approach where we noticed that while the alleles from both ICMR1004 and ICMR1029 which contributed to most of the crop production related traits under WW, the allele from 1029 specifically contributed to the most of the crop production related traits in severe water stress. This, at minimum, means that the processes which contributed to plant growth in WW and SS were very different and traits allowing later plant growth in SS were related to traits permitting water saving at early/vegetative stages. Also, PCA confirmed that GY was tightly related to biomass accumulation capacity (total dry weight (TOTDW) and tillers (TNO)) in WW but their importance for GY was considerably weakened in SS.

From the results of co-localisation, we could observe that under WW, TNO has the QTLs at R1, R2 and R3 with the alleles contributed from both the parents (ICMR1029 and ICMR1004) whereas under SS, the alleles for TNO was only contributed by the recurrent parent (ICMR1004). Therefore the QTLs for TNO under SS were not as useful as under WW.

555 The results of PCA shows that GY was closely related to TNO under WW but not under SS.
556 One possible reason could be the effect of SS on tiller grain filling i.e. though the tillers
557 initiation occurred at early plant development stage (under WW), the stress imposition at later
558 plant development stage probably stopped or hampered the grain filling of these tillers. In
559 other words, producing tiller would be a worthy strategy in situation where there is no water
560 limitation, but a drawback under water limitation where the investment in tiller would not be
561 rewarded by grain produced from these tillers.

562 Apart of tiller contribution to GY in different conditions, very interesting was the dissection
563 of genetics underlying the plant tillering capacity. Tiller number was consistently determined
564 by several QTLs (R1, R2 and R3) where some were common with QTLs regulating early
565 canopy development and related traits (CS and PH) i.e. R1 (191-205) and R3 (236-239,
566 Fig.3). This is consistent with previous findings documenting that tillering propensity is
567 determined by the main stem carbon-supply/demand status during early plant growth which
568 means that plants with smaller canopy (consistent with initial co-localization in R1) are likely
569 to attain higher carbon S/D ratio and tiller more (Kim et al. 2010 a, b, Alam et al. 2014).
570 Same authors also indicated that tillering propensity depends on hormonal signalling which is
571 independent of early canopy growth.

572 **Crop improvement strategy**

573 Our study clearly demonstrated that some traits which support crop production in one
574 environment might bring production penalty in another (Tardieu 2012, Vadez et al. 2013b,
575 Kholova et al. 2013). In our study we anticipated that in environments with unlimited water
576 access, crop production could be increased by improvement of crop production potential.
577 Traits associated with “crop production potential” were GY that was determined by biomass,
578 itself in turn was determined by tillers. These tillers were determined by canopy development

which was in turn determined by transpiration. All these traits were under-laid by strong action of R1, R2, R3 and R4 genetic regions.

In contrary, in severely water limited environments, where water can be stored in the soil profile, we showed that crop production might benefit from less vigorous growth which is associated with traits like smaller canopy (Vadez et al. 2013b) or restricted transpiration rate by which the water saved during pre-anthesis could be used during the post anthesis for grain filling (Vadez et al. 2013a). In this, under SS, most of the traits on crop production were contributed by the parent ICMR1029. However, to use such traits in crop improvement programs, one has to rigorously quantify the possible site-specific frequency of such environmental occurrence and benefits/trade-offs associated with these traits in these circumstances (e.g. using long series of multi-location trials or crop models Vadez et al. 2013c, Kholova et al. 2014).

Overall, this study revealed that crop production related traits were linked to water –use related traits and so more attention should be paid for water-use related traits in order to achieve success in crop production under water deficit environment. The preferred ideotype would be targeting four genetic regions that covers most of the QTLs associated with canopy development, water-use and crop production and the alleles that favors the grain filling under specific environment and conditions.

Author contribution statement

MT, JK, KS, VV and BR performed phenotyping. DS genotyped the molecular markers with RY. CTH developed the fine mapping population. RB prepared the files of configuration (LeasyScan experiments) and randomization (LeasyScan, Lysimeters and Field experiments). MT carried out the analysis, prepared the tables and figures and wrote the manuscript. JK and

VV conceived the study, provided advice on analysis and interpreting the results. Both JK and VV reviewed the paper with TT.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends

Figure 1 Relationships between a) grain yield ($gplant^{-1}$) and harvest index b) grain yield ($gplant^{-1}$) and transpiration efficiency (gkg^{-1}) c) residual yield not explained by the harvest index (calculated from regression equations of Fig. 1a) and transpiration efficiency and d) residual yield not explained by the harvest index and post anthesis water extraction ($gplant^{-1}$). Data are means of five replicated plants per genotype under severe water stress treatment. * and ** indicates the significant difference statistically at $p<0.05$ and $p<0.001$ respectively.

Figure 2 Range of variation obtained for different traits: a) 3dimensional leaf area (cm^2; WW) b) transpiration at 64 DAS ($gweek^{-1}$; SS), c) total dry weight ($gplant^{-1}$; WW) and d) grain yield ($gplant^{-1}$; MS). . * and ** indicates the significant difference statistically at $p<0.05$ and $p<0.001$ respectively.

Figure 3 QTL co-localisation of the plant low level organisation traits (canopy development and water-use related traits) and high level organisation traits (biomass and grain production related traits) on the 17 polymorphic markers region (highlighted in yellow colour) of linkage group2 (LG02). The position of the QTLs mapped from cartographer CIM (Composite Interval Mapping) method for the phenotypic traits were indicated in either in red (positive additive effect of the alleles from 1029) or green (positive additive effect of the alleles from 1004) and the numbers in the cell represents the LOD values. WW-well-watered; MS-mild water stress; SS-severe water stress. The environment used for phenotyping each trait were

768 indicated by suffix letters; P-Pot culture; LS-LeasyScan; L-Lysimeter and F-field. Refer to
769 Table 1 for the acronym of the traits.

770 **Figure 4** Principal component analysis for a) field traits under WW b) field traits under SS c)
771 grain yield from field (SS) and traits from Lysimeter (SS) d) grain yield from field (WW) and
772 traits from LeasyScan (WW); e) grain yield from field (SS) and traits from pot culture (WW)
773 and f) early water extraction ((T36DAS and T41DAS) from Lysimeter SS)) and canopy
774 development related traits (3DLA, PLA, 3DGR, PGR, CS and PH) from LeasyScan under
775 WW. The oval shape in blue encompass the closely related traits. The suffix to the trait code
776 indicate the environment (F-Field; L-Lysimeter; LS-LeasyScan and P-pot) followed by year
777 of phenotyping and water stress treatment (WW-well watered; MS-mild water stress and SS-
778 severe water stress). Refer to Table 1 for the acronym of the traits.