

RESEARCH PAPER

Transpiration difference under high evaporative demand in chickpea (*Cicer arietinum* L.) may be explained by differences in the water transport pathway in the root cylinder

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ABSTRACT

- Terminal drought substantially reduces chickpea yield. Reducing water use at vegetative stage by reducing transpiration under high vapor pressure deficit (VPD), *i.e.* under dry/hot conditions, contributes to drought adaptation. We hypothesized that this trait could relate to differences in a genotype's dependence on root water transport pathways and hydraulics.
- Transpiration rate responses in conservative and profligate chickpea genotypes were evaluated under increasing VPD in the presence/absence of apoplastic and cell-to-cell transport inhibitors.
- Conservative genotypes ICC 4958 and ICC 8058 restricted transpiration under high VPD compared to the profligate genotypes ICC 14799 and ICC 867. Profligate genotypes were more affected by aquaporin inhibition of the cell-to-cell pathway than conservative genotypes, as measured by the root hydraulic conductance and transpiration under high VPD. Aquaporin inhibitor treatment also led to a larger reduction in root hydraulic conductivity in profligate than in conservative genotypes. In contrast, blockage of the apoplastic pathway in roots decreased transpiration more in conservative than in profligate genotypes. Interestingly, conservative genotypes had high early vigour, whereas profligate genotypes had low early vigour.
- In conclusion, profligate genotypes depend more on the cell-to-cell pathway, which might explain their higher root hydraulic conductivity, whereas water-saving by restricting transpiration led to higher dependence on the apoplastic pathway. This opens the possibility to screen for conservative or profligate chickpea phenotypes using inhibitors, itself opening to the search of the genetic basis of these differences.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second most important food legume worldwide (FAOSTAT 2013). Chickpea production areas are mainly in arid and semi-arid zones, where end of season drought (terminal drought, *i.e.* stress in the post-flowering period, and critically at the grain filling stage) is a major constraint. Such constraints reduce yield, typically by more than 50% (Leport *et al.*, 1999, 2006; Kumar & Abbo, 2001). Crop adaptation to water limitation is a balance between maximizing water use and ensuring a crop has sufficient water to complete its cycle (Vadez *et al.*, 2013a). One such adaptation involves controlling plant water loss during vegetative growth, even when water is plentiful, by restricting transpiration under high vapor pressure deficit (VPD), *i.e.* conditions combining high temperature and low relative humidity. Conserving soil moisture for the later grain filling stage is crucial to increase crop yield under terminal water stress (reviewed in Vadez *et al.*, 2013a). A mechanism for reducing transpiration under high VPD has been described for chickpea (*Cicer arietinum* L.;

Zaman-Allah *et al.*, 2011a) and other species, such as pearl millet (*Pennisetum glaucum* L.; Kholová *et al.*, 2010), sorghum (*Sorghum bicolor* L.; Gholipour *et al.*, 2010), soybean (*Glycine max* (L.) Merr; Fletcher *et al.*, 2007; Gilbert *et al.*, 2011), wheat (Schoppach & Sadok 2012), peanut (*Arachis hypogaea* L.; Devi *et al.*, 2010), cowpea (*Vigna unguiculata* L.; Belko *et al.*, 2013) and in maize (*Zea mays* L.; Yang *et al.*, 2012; Gholipour *et al.*, 2013). This water conservation strategy has been shown to be directly linked to increased crop production under water-limited conditions (Zaman-Allah *et al.*, 2011a; Messina *et al.*, 2013; Vadez *et al.*, 2013b, 2014; Kholová *et al.*, 2014). Reduced extraction of soil water by deeper roots during the vegetative stage is expected to retain more water in the soil profile for extraction at later growth stages, *e.g.* grain filling, when water becomes scarce. This strategy makes sense for the heavy clay and often deep soils in which chickpea is cultivated in India.

Restriction of transpiration under high VPD might be related to differences in the way in which water is channeled through the plant. It is generally agreed that there are two major sites of resistance to water flow through plant tissues: (i)

at canopy level, *i.e.* at the leaf surface where the stomata and cuticle control water flow without restriction through the xylem; and (ii) at the root system level, *i.e.* through the root cylinder (Steudle, 1994; Steudle & Peterson, 1998). According to Steudle & Frensch (1996), a composite model for root water uptake involves three pathways: apoplastic, symplastic (mediated by plasmodesmata) and trans-cellular (mediated through cell and vacuole membranes so that passage of water through the trans-cellular pathway involves crossing two plasma membranes per cell layer). The last two are often aggregated and called the cell-to-cell pathway. The resistance of each pathway differs and the predominance of one path over another may depend on the driving force and on the respective resistance of each pathway (Miyamoto *et al.*, 2001). Water transport in the apoplastic compartment is mainly driven by hydrostatic forces, while the cell-to-cell pathway involves both osmotic and hydrostatic gradients. Hydrostatic forces are caused by negative pressure in the xylem as a consequence of plant transpiration, in which case water is pulled towards the xylem to compensate for these negative pressures. Osmotic forces result from the pumping of solutes in the roots which creates a positive root pressure that contributes to push water into the xylem. Based on evaporative demand from the shoot, the composite transport model would allow for adjustment between the apoplastic and cell-to-cell pathways (Ranathunge *et al.*, 2003, 2005).

It should be noted that the separation between the cell-to-cell and apoplastic pathways remains a theoretical representation of water movement in root cylinders, since apoplastic water also needs to go through the cell-to-cell pathway, at least in the endodermis where the Casparian band and suberization block apoplastic water flow. Therefore, what we call the ‘apoplastic’ pathway in this paper also implies that water may traverse aquaporins at some point. There is numerous evidence that the cell-to-cell pathway involves water transport through aquaporins, a family of major intrinsic proteins (MIP) known for their ability to facilitate water flow (Javot & Maurel, 2002; Fricke & Chaumont, 2006; Hachez *et al.*, 2006; Maurel, 2007; Katsuhara *et al.*, 2008). Therefore, the cell-to-cell pathway could be rapidly tuned up or down, depending on plant need. As such, the cell-to-cell pathway offers additional flow control through the activity of aquaporins. However, the extent to which either pathway contributes to root water channelling in various species, genotypes and in response to environmental pressures, as much as the two pathways can be distinguished, is still not understood. In addition, apoplastic and cell-to-cell water transport pathways may differ in terms of hydraulic conductivity (Steudle, 2000; Javot & Maurel, 2002; Aroca *et al.*, 2005). Therefore, the relationship of these two pathways with traits controlling plant water use and with possible differences in these traits among genotypes are investigated here. This was tested by either inhibiting aquaporin-mediated water flow in the cell-to-cell pathway using aquaporin inhibitors, or by blocking the water flow in the apoplast using a perfusion technique (Ranathunge *et al.*, 2004) in contrasting chickpea genotypes.

The objective of this study was to test the hypothesis that chickpea genotypes with contrasting transpiration responses to increasing VPD may respond differently to the different water transport pathways in the root cylinder. Therefore this work aimed to: (i) confirm the transpiration response to VPD differences among several genotypes; (ii) compare their cell-to-cell

(aquaporin-mediated) and apoplastic water flow by examining the response of transpiration to chemical inhibition of either pathway; and (iii) measure root hydraulic conductivity after aquaporin inhibition.

MATERIAL AND METHODS

Plant material and growth conditions

Four chickpea genotypes with contrasting transpiration response to high VPD were selected. The salient features of the selected genotypes are described in Table 1. Two genotypes (ICC 867 and ICC 14799) showed profligate transpiration under high VPD, whereas one (ICC 8058) had conservative transpiration under high VPD (Zaman-Allah *et al.*, 2011a). Genotype ICC 4958, a popular drought-tolerant genotype, was also expected to have conservative transpiration under high VPD, as it has low stomatal conductance under high VPD in outdoor experiments (Zaman-Allah *et al.*, 2011b). Other information on these genotypes was obtained from the ICRI-SAT mini-core collection under terminal drought stress

Table 1. Salient features of the four selected chickpea genotypes (days to 50% flowering, days to maturity, drought tolerance) with contrasting plant water saving characteristics (profligate and conservative). Data on days to flowering (DF), days to maturity (DM) and drought tolerance are mean of 3 years of field experiments (Krishnamurthy *et al.*, 2010). Data on shoot components (leaf size and total leaf area) were from soil-grown plants during the transpiration assessment in Experiment 1. Shoot biomass and leaf area index are from soil-grown plants during a LeasyScan phenotyping experiment (Sivasakthi *et al.* – unpublished data). Root components were from hydroponically grown plants in the root hydraulic conductivity experiments (2–4).

trait	ICC 867	ICC 14799	ICC 4958	ICC 8058
description	(Profligate)	(Profligate)	(Conservative)	(Conservative)
Shoot components				
Leaf size	Smaller	Smaller	Larger	Larger
Total leaf area (cm ² plant ⁻¹)	85	105	180	150
Shoot biomass (g plant ⁻¹)	2.63	2.60	3.89	3.51
Leaf area index (LAI)	0.52	0.51	0.71	0.66
Root components				
Root surface area (cm ²)	51	55	82	71
Root volume (cm ³)	1.9	2	3	2.6
Root dry weight (g)	0.09	0.12	0.14	0.17
Days to 50% flowering (DF)	44	48	37	47
Days to maturity (DM)	88	90	82	95.6
Drought tolerance	High tolerance	Tolerant	Moderate tolerance	Sensitive

conditions in the field for 3 years (Krishnamurthy *et al.*, 2010) (Table 1).

Plants were grown in a glasshouse, either in pots filled with soil or in hydroponic conditions (see below for details). Day/night temperatures and relative humidity (RH, %) were, on average, 28/22 °C and 70/90%, respectively, under natural a photoperiod. Details of this experiment are reported in Table 2.

Transpiration response to increasing VPD

Experiment 1 was carried out to test and confirm whole plant transpiration response to increasing VPD of the above genotypes 32 day after sowing (DAS), when plants were still at the vegetative stage. Plants were grown in plastic pots (3 l; 19.0-cm diameter × 15.24-cm high) filled with 1.5 kg of Vertisol, collected from the ICRISAT farm, and fertilized with DAP (diammonium phosphate) at 0.3 g·kg⁻¹ soil. Each pot received 0.1 g carbofuran applied to the surface of the soil a day before sowing to prevent soilborne pests. Seeds were treated with

thiram (fungicide) to avoid fungal contamination. Three seeds were sown in each pot and a rhizobium inoculum (Strain No: IC 2002) was added to each pot to ensure nodulation. Two weeks after sowing, plants were thinned to one plant per pot. Plants were grown under well-watered conditions for 32 days (vegetative stage).

A plastic sheet was put on top of the soil in each pot and then a 2-cm layer of plastic beads was poured on top of the sheet to limit soil water evaporation. Eight replicate plants were used for each genotype. Plants were moved to the controlled environment growth chamber (Conviron-PGW36; Controlled Environments, Winnipeg Manitoba, Canada, http://www.conviron.com/sites/default/files/PGW36%20Data%20Sheet_1.pdf) about 40 h before assessing plant transpiration response to increasing VPD. The plants were acclimatized for an entire day to growth chamber conditions, *i.e.* light period (06:30–18:30 h; 1.8 kPa, *i.e.* 31 °C, 60% RH, 450 μmol·m⁻² s⁻¹ photosynthetic photon flux density) and night time VPD (19:30–05:30 h; 0.9kPa, *i.e.* 27 °C, 75% RH). The day before the experiment, pots were irrigated and left to drain overnight to reach field

Table 2. Description of experiments, material tested, plant growth system, plant growth location, treatment, plant part investigated and trait measured

exp.no	genotype	experiment	plant growth system - Environment	plants part investigated a trait measured - Experiment location	concentration of inhibitors	no. replicates per genotype per treatment	plant age when tested	VPD (kPa) in experiment
1	ICC 14799 ICC 867 ICC 4958 ICC 8058	TR response to increasing VPD	Pots with black soil (Vertisol) - glasshouse	whole plant, TR (mg·H ₂ O cm ⁻² min ⁻¹) - growth chamber	–	8	30	(0.7–4.21)
2	ICC 14799 ICC 867 ICC 4958 ICC 8058	Aquaporin inhibition	Hydroponics - Glasshouse	whole plant, level of aquaporin inhibition - Growth chamber	1 mM H ₂ O ₂	4 control 4 treatment	25	3.1
3	ICC 14799 ICC 867 ICC 4958 ICC 8058	Aquaporin inhibition	Hydroponics - Glasshouse	whole plant, level of aquapoein inhibition - Growth chamber	100 μM HgCl ₂ and 200 μM HgCl ₂	4 control 4 treatment	25	3.1
4	ICC 14799 ICC 867 ICC 4958 ICC 8058	Apoplastic inhibition	Hydroponics - Glasshouse	whole plant, level of apoplastic inhibition - Growth chamber	1 mM K ₄ [Fe(CN) ₆] and 0.5 mM CuSO ₄	8 control 8 treatment	26	3.1
5	ICC 14799 ICC 867 ICC 4958 ICC 8058	Root hydraulic conductivity after aquaporin inhibition	Hydroponics - Glasshouse environment	De-topped roots, Root hydraulic conductivity (mg·H ₂ O cm ⁻² min ⁻¹ MPa ⁻¹) - Glasshouse	100 μM HgCl ₂	8 control 8 treatment	23	1–2.5

capacity. Pots contained 300–350 ml water available for plant transpiration, which was in excess of daily plant transpiration so that no water stress was expected during the experiment. On the day of the experiment, VPD was gradually increased (from 0.9 kPa to 4.21 kPa; Table S1) at 60-min intervals during seven consecutive hours (08:00–15:00 h). Within each VPD level, plants were weighed gravimetrically every 60 min using 0.01 g precision scales (KERN 3600-2N; Kern & Sohn, Balingen, Germany), which took about 10 min. During this time, the VPD was increased to the next level. A data logger for air temperature and relative humidity (Lascar Electronics, UK) was positioned within the plant canopy in the growth chamber. Increases in VPD were achieved by simultaneously increasing the temperature (from 27 to 37 °C) and decreasing the relative humidity (from 75 to 33%), mimicking natural conditions outdoors, and within ranges that the crop faces under natural conditions (see Table S1). Plants were harvested at the end of the transpiration measurement to measure leaf area (LI-3100C area meter; LI-COR Biosciences, USA). The individual plant transpiration rates (TR) were obtained by dividing transpiration values by leaf area.

Transpiration response to aquaporin inhibition of whole plants using H₂O₂ and HgCl₂

Experiments 2 and 3 were carried out to test the transpiration response to a set of inhibitors of the cell-to-cell or apoplastic pathway and investigate plant hydraulic characteristics. Plants were grown in a hydroponic system installed in a glasshouse (climate conditions are described above). Seeds were treated and sown in sand wetted with nutrient solution. Rhizobium inoculum (Strain No: IC 2002) was added to ensure nodulation. A week later, seedlings were transferred to 250 ml Erlenmeyer conical flasks containing modified Hoagland nutrient solution. The composition of the solution was 1 mM MgSO₄, 0.92 mM K₂SO₄, 0.75 mM CaCl₂·2H₂O, 0.25 mM KH₂PO₄, 0.04 mM Fe-EDTA, 5 mM urea and micronutrients (2.4 μM H₃BO₃, 0.9 μM MnSO₄, 0.6 μM ZnSO₄, 0.62 μM CuSO₄, 0.6 μM Na₂MoO₄). The pH of the nutrient solution was adjusted between 6.0 and 6.2. Seedlings were passed carefully through the hole of a rubber stopper that fitted tightly to the flask aperture and the hypocotyle was fixed with cotton to prevent the seedling from slipping through. The glass flasks were covered with two layers of paint: first a black layer to ensure darkness in the rooting medium and prevent algal growth; then a layer of white paint to reflect excess sunlight and prevent heating of the flask. Aeration was continuously supplied to roots *via* a spaghetti tube distribution system with a flow rate above 500 ml·air·min⁻¹. Water was replaced daily to compensate for any water loss and nutrient solution in the flasks was changed once every 3 days.

To inhibit aquaporin-mediated water flow, aquaporin inhibitors (HgCl₂ and H₂O₂) have been used in several reports (HgCl₂: Sadok & Sinclair, 2010; Devi *et al.*, 2012, 2016; H₂O₂: Henzler *et al.*, 2004; Ye & Steudle, 2006). Mercury (Hg⁺ ions) reacts with the sulphohydril group of cysteine residues within the aquaporin through covalent modifications that change the conformation of the protein leading to inhibition of water transport (Niemietz & Tyerman, 2002). Hydrogen peroxide is involved in oxidative gating of hydroxyl radicals, resulting in chemical modification of aquaporins, a mechanism reported in

maize and chara plants (Henzler *et al.*, 2004; Ye & Steudle, 2006). In addition, H₂O₂ regulates the gating of aquaporins through phosphorylation/dephosphorylation (Boursiac *et al.*, 2008). In experiments 2 and 3 (Table 2), following earlier work (Tharanya *et al.*, 2018), 28-day-old (vegetative stage) hydroponically grown plants were transferred to a growth chamber about 40 h before the experiment to allow acclimation. After acclimation (1.8 kPa, *i.e.* 31 °C and 60% RH, 450 μmol·m⁻²·s⁻¹; see below for further details) for 1 day, plant transpiration was measured over a 7-h period, starting at 08:00 h under a constant and relatively high VPD (3.1 kPa; *i.e.* 35 °C, 45% RH, 450 μmol·m⁻²·s⁻¹). Transpiration was measured automatically with 0.01 g precision scales every 10 min. Transpiration of each plant was measured for 3–4 h without any treatment and then the response of transpiration to aquaporin inhibitor treatments was tested by exposing whole plant roots to 1 mM H₂O₂ applied to the nutrient solution. Transpiration was recorded for 4 h following this treatment. For each genotype, four untreated (control) and four H₂O₂-treated plants were analysed. The same protocol was applied to test the transpiration response to two HgCl₂ treatments (100 or 200 μM HgCl₂; Experiment 3).

After treating plants with the aquaporin inhibitor (1 mM H₂O₂), we tested whether differences in transpiration response to cell-to-cell pathway inhibition could be proxied using canopy temperature measurements. Thermal images (infrared, IR) were taken 2 h after treatment with H₂O₂, using an IR Flex-Cam S (Infrared Solutions, Plymouth, MN, USA) with a sensitivity of 0.09 °C and accuracy of ±2%. Images were taken from three plants for each genotype and treatment. SmartView 2.1.0.10 software (Fluke Thermography Everett, WA, USA) was used for analysis of the thermal images and estimation of canopy temperatures.

Transpiration response to apoplast inhibition using precipitates of CuSO₄

Experiment 4 (Table 2) was carried out to test transpiration response to an inhibitor of the apoplastic pathway and investigate plant hydraulic characteristics in 28-day-old hydroponically-grown plants. The apoplastic blockage consisted in exposing plant roots to insoluble salts to block the apoplastic pathway. This consisted in first treating roots with a solution of 1 mM K₄[Fe(CN)₆], before changing the solution after 1 h and treating the roots with a solution of 0.5 mM CuSO₄. The reaction between CuSO₄ and K₄[Fe(CN)₆] produced rusty-brown, insoluble crystals (precipitates) of Cu₂[Fe(CN)₆] or Cu[CuFe(CN)₆] (Fig. 4D), which obstructed the apoplast lumen (Daniels *et al.*, 1994). Different combinations of exposure duration to K₄[Fe(CN)₆] (1 mM) were initially tested to determine the optimum time for development of insoluble crystals to significantly restrict the apoplastic pathway. The standardized concentration was 1 mM K₄[Fe(CN)₆] and 0.5 mM CuSO₄. Transpiration was measured over 1 h without any treatment, followed by 3 h of transpiration measurement in the presence of 1 mM K₄[Fe(CN)₆], and finally 3 h transpiration measurements following addition of 0.5 mM CuSO₄. A 1 mM K₄[Fe(CN)₆] treatment was used as control, as this had no effect on transpiration (Tharanya *et al.*, 2018). Eight replicate plants per genotype and treatment combination were used. Finally, root samples were collected around 30 mm from the apex and

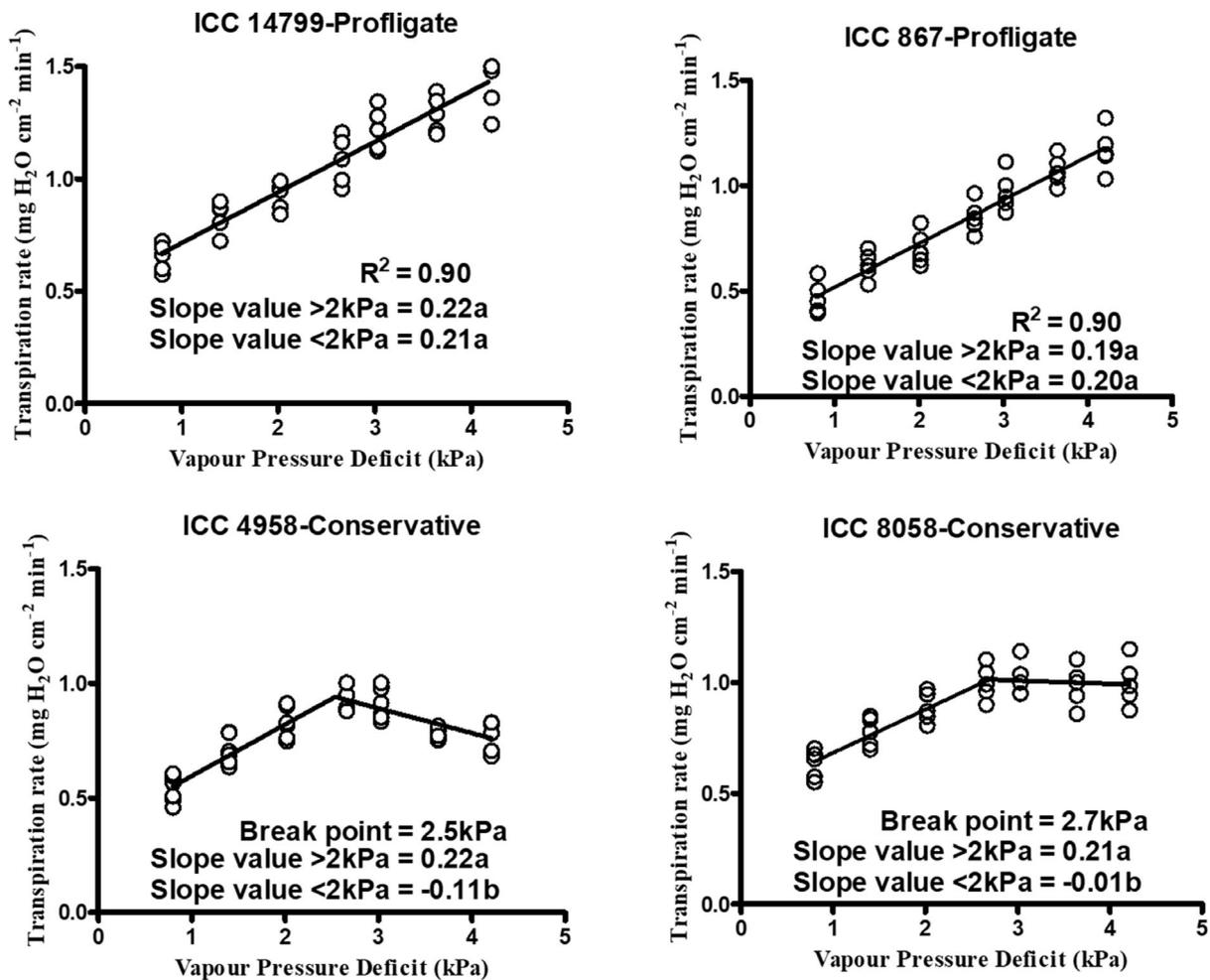


Fig. 1. Response of transpiration ($\text{mg}\cdot\text{H}_2\text{O}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$) to increasing VPD in four chickpea genotypes [ICC 867 and ICC 14799 (profligate); ICC 4958 and ICC 8058 (conservative)]. Plants were grown in black soil (Vertisol) and tested at vegetative stage (32 days old). Each data point represents an individual plant response to VPD. Mean slope values (>2 kPa and <2 kPa) followed by different letters indicate significant differences using Tukey's test. *** $P < 0.001$, ns no significant difference in slope >2 kPa and <2 kPa. See also Table 3 for details.

stained with 0.05% Toluidine blue at room temperature for 2 min. Free-hand root cross-sections were examined under a light microscope and photographed using a digital camera to confirm the presence of nanoparticles in the root cortex and especially in the root apoplast.

Hydraulic conductivity of detached roots in response to $100\ \mu\text{M}$ HgCl₂ treatment

In experiment 5 (Table 2), hydroponically-grown plants were used to measure root hydraulic conductivity in a pressure chamber (PMS instruments, Corvallis, OR, USA) using a protocol similar to that used for barley (Tazawa *et al.*, 1997), tomato (Maggio & Joly, 1995), wheat (Zhang and Tyerman, 1999) and pearl millet (Tharanya *et al.*, 2017). This measurement was done under glasshouse conditions in plants previously treated with an aquaporin inhibitor or kept under non-treated conditions. The shoot was cut with a razor blade and the detached root, bathed in water, was carefully placed in the pressure chamber and sealed using silicon glue and polyvinylsiloxane (Coltene President, Switzerland) to prevent pressure

leakage. Pressure was applied in the root medium at 0.1, 0.2 or 0.3 MPa, successively for 15 min each. The root exudate (xylem sap) was collected every 5 min at the cut surface using pre-weighed Eppendorf cones stuffed with tissue paper (Kimtech Science, Ontario, Canada). Sap collection was done three times at each pressure, and the next pressure was applied once a constant exudation rate was reached. The average value of the three exudation samples was normalized to root surface area, pressure and time. The root surface area was estimated by scanning with a Shimadzu scanner and analysed with Winrhizo software (Winrhizo, Regent, Canada).

Statistical analysis

In all experiments, transpiration data were normalized for the leaf area. For that, individual plant transpiration rate (TR, $\text{mg}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$) values were calculated by dividing transpiration values (T, $\text{mg}\cdot\text{min}^{-1}$) from the consecutive pot weighings, by leaf area (in cm^2), measured at the end of each transpiration response experiment. Transpiration rate values were then normalized as described earlier (Kholová *et al.*, 2010) to facilitate

genotype and treatment comparisons. For the inhibition experiment, TR data were double normalized to the non-treated control; first by dividing individual TR data by mean TR of the control (TR ratio; TRR), then by dividing these TRR values by mean of TRR values before inhibitor treatment (Normalized TRR, NTRR), following earlier work (Sivasakthi *et al.*, 2017; Tharanya *et al.*, 2018). The NTRR data from inhibition of water transport pathways (cell-to-cell and apoplastic inhibition), transpiration rate response to increasing VPD data and root hydraulic conductivity data were analysed with the statistical package CoStat (version 6.204; Cohort Software, Monterey, CA, USA). One-way ANOVA was carried out to test for genotypic differences between treatments and genotypes. Means were compared using Tukey-Kramer test and LSD (at $P = 0.05$).

Two-way ANOVA was used to evaluate the effect of treatment and genotype, or genotype groups (conservative and profligate) and their interactions. For these analyses, the following data were used: (i) transpiration rate response to increasing VPD (Expt.1), replicated slope value data were used to test difference between slope value >2 kPa and <2 kPa; (ii) the inhibition experiment used the replicated plant mean data of NTRR once stable transpiration was reached after inhibition; (iii) differences in canopy temperature data before and after inhibition; (iv) root hydraulic conductivity used normalized root exudate data after normalizing against root surface area, pressure and time ($\text{mg}\cdot\text{H}_2\text{O}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}\cdot\text{MPa}^{-1}$) of both control and inhibited plants of each genotype and replicate. To fit data on transpiration rate response of each genotype to increasing VPD in the growth chamber, we applied a segmented linear regression (model $Y_1 = \text{Slope}_1 \times X + \text{Intercept}_1$ and $Y_2 = \text{Slope}_2 \times X + \text{Intercept}_2$) or a linear regression (model $Y_1 = \text{Slope}_1 \times X + \text{Intercept}_1$); these algorithms fit the best model depending on the data, giving a 95% confidence interval, significance at $P > 0.05$, and comparing slopes (Medina *et al.* 2019). This analysis was performed with Graph Pad Prism version 6 (Graph Pad Software, CA, USA).

RESULTS

Transpiration response to increasing VPD in the growth chamber

Upon VPD increase, TR increased in all tested genotypes, but the genotypes differed in the magnitude of TR response (rate of TR change). ICC 867 and ICC 14799 had a linear increase in TR with no restrictions throughout the whole range of tested VPD (Fig. 1), whereas ICC 4958 and ICC 8058 had TR

restriction at VPD above 2 kPa, and then a decrease in transpiration beyond the transpiration breakpoint. VPD breakpoints of 2.53 and 2.69 kPa were found for ICC 4958 and ICC 8058, respectively (Table 3, Fig. 1). Therefore, ICC 4958 and ICC 8058 had significantly lower TR than ICC 867 and ICC 14799 under high VPD conditions (>2 kPa) conditions (Fig. 1).

The slope values for ICC 4958 and ICC 8058 were 0.22 and $0.21\text{ mg}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$ before the VPD breakpoints, and these were not significantly different from the slope values for ICC 14799.

Transpiration response to aquaporin inhibitor treatment of whole plants with H_2O_2 or HgCl_2

Transpiration was measured for 3–4 h prior to applying the aquaporin inhibitors treatment to the roots and remained stable over time. In all genotypes, the pattern of aquaporin inhibition was similar: H_2O_2 had a consistently weak effect, followed by $100\ \mu\text{M}$ Hg, while $200\ \mu\text{M}$ Hg uniformly had the strongest effect on the normalized transpiration rate ratio (NTRR) (Fig. S1). The analysis of NTRR response to treatment with $1\ \text{mM}$ H_2O_2 revealed that, after 20 min of exposure to the inhibitor, NTRR decreased faster in the profligate genotypes (ICC 867 and ICC 14799) than in the conservative genotypes (ICC 4958 and ICC 8058) (Fig. 2A–C). The maximum inhibition occurred about 120 min after exposure to H_2O_2 , with a NTRR decrease of profligate genotypes of 25% in ICC 867 and 30% in ICC 14799 (Fig. 2A; Fig. S1). In contrast, conservative genotypes showed a significantly lower decrease in transpiration, *i.e.* 8% in ICC 4958 and 15% in ICC 8058 (Fig. 2A; Fig. S1). After 120 min of exposure to $1\ \text{mM}$ H_2O_2 , NTRR recovered to nearly 100% in ICC 4958 and about 88% in ICC 8058 at about 270 min after treatment (Fig. 2A). Whether there could have been acclimation over time is not known and was not assessed here for the long term (*i.e.* several hours after treatment) in this experiment. The inhibition differences between genotypes was statistically significant at $P < 0.01$ (Fig. S1).

Compared to H_2O_2 , HgCl_2 induced higher inhibition of NTRR in all tested genotypes (Fig. 2A–C). The NTRR decreased in a dose-dependent way, with a stabilization of the NTRR at about 90–120 min after exposure to the inhibitor (Fig. 2B,C; Fig. S1). With $100\ \mu\text{M}$ HgCl_2 , NTRR decreased significantly more ($P < 0.001$) in the profligate than in the conservative genotypes (Fig. S1). In detail, NTRR decreased by about 60% in relation to transpiration of the untreated control (% representing mean of stabilized data points) in ICC 867 and ICC 14799 (profligate) about 100 min after treatment. In

Table 3. Mean transpiration rate (over the whole VPD rang in Experiment 1), break point values (when significant) and slope values above and below 2 kPa for transpiration rate (TR) in response to increasing VPD, assessed in four chickpea genotypes (ICC 4958 and ICC 8058 – conservative, ICC 867 and ICC 14799 – profligate) with contrasting TR response to VPD. Mean values (>2 kPa and <2 kPa) followed by different letters indicate significant differences with Tukey's test

genotype	mean TR $\text{mg}\cdot\text{H}_2\text{O}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$	break point kPa	slope value >2 kPa		slope value <2 kPa		R^2
			$\text{mg}\cdot\text{H}_2\text{O}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$	SE	$\text{mg}\cdot\text{H}_2\text{O}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$	SE	
ICC 4958	0.781	2.53	0.22a	0.01	–0.11c	0.01	0.77
ICC 8058	0.899	2.69	0.21a	0.01	–0.01b	0.04	0.80
ICC 14799	1.096	No break point	0.22a	0.01	0.21a	0.01	0.90
ICC 867	0.809	No break point	0.19a	0.003	0.20a	0.01	0.90

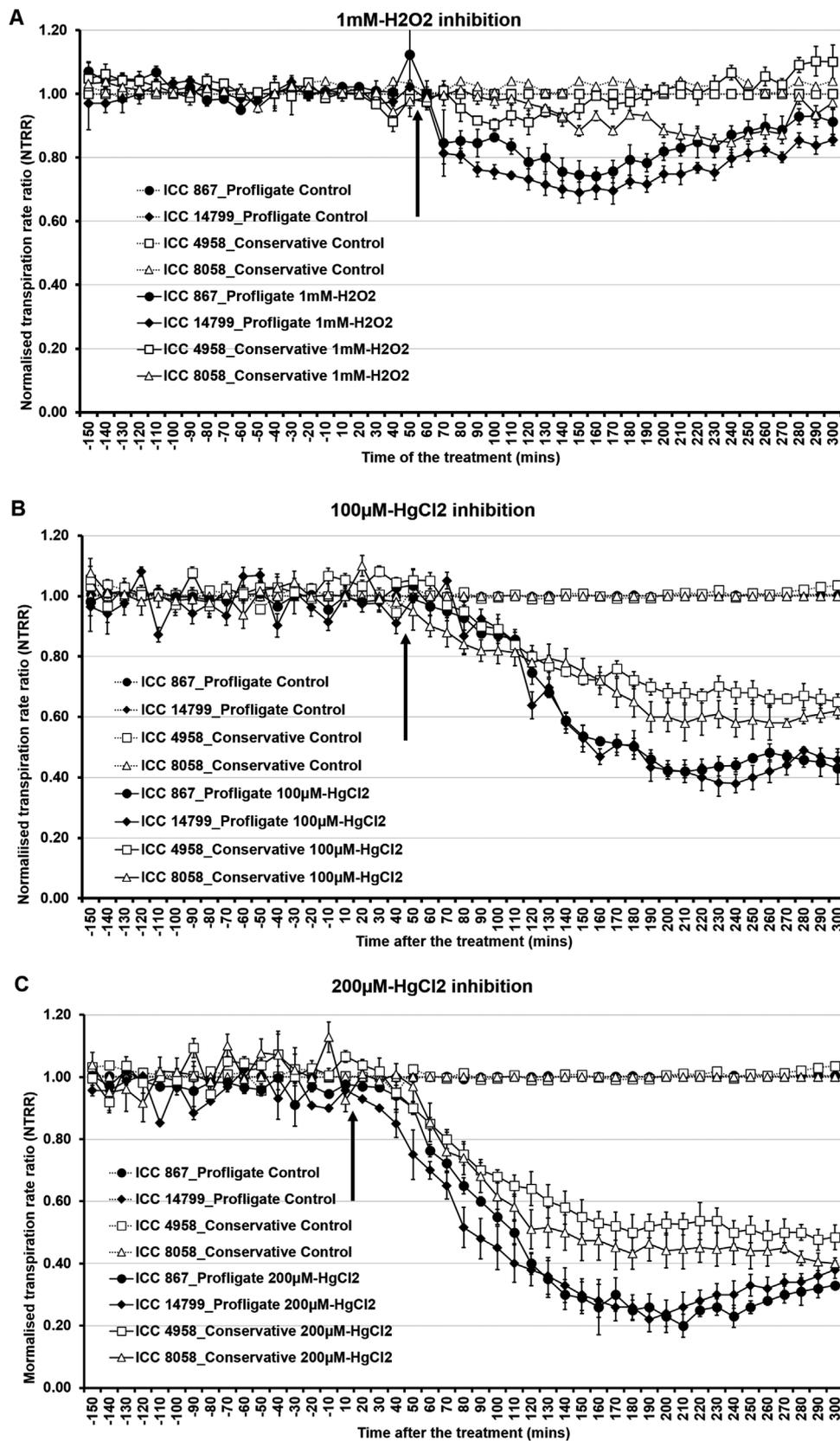


Fig. 2. Decline in normalized transpiration rate ratio (NTRR) upon aquaporin inhibitor treatment in four chickpea genotypes with contrasting transpiration response to high VPD: 1 mM H₂O₂ (A), 100 µM HgCl₂ (B), 200 µM HgCl₂ (C). Genotypes ICC 867 and ICC 14799 are profligate (filled circle and filled diamond) and ICC 4958 and ICC 8058 are conservative (open square and open triangle). Plants were grown in hydroponics and tested at vegetative stage (26 days old). Each data point (n = 4) represents NTRR means ± SE of four replicates per genotype per treatment. The arrow represents the time of application of treatment.

contrast, in ICC 8058 and ICC 4958 (conservative) the NTRR reduction was about 25% (Fig. S1). For the 200 μM HgCl_2 treatment, the decrease in NTRR was more rapid than with 100 μM , particularly for the profligate genotypes ICC 867 and ICC 14799, and NTRR decreased significantly more ($P < 0.001$) in the profligate than in the conservative genotypes (Fig. S1). The 200 μM HgCl_2 treatment led to a 65–70% reduction in transpiration measured at 60 to 150 min after Hg treatment in the profligate genotypes (Fig. 2B,C; Fig. S1). In contrast, the conservative genotypes ICC 4958 and ICC 8058 only had a 40–45% NTRR reduction (Fig. S1).

Hydraulic conductivity of detached roots in response to the aquaporin inhibitor (100 μM HgCl_2)

Root hydraulic conductivity measurements were made over several days and each was at a different time of day. Figure S2 shows that morning root hydraulic conductivity was higher than midday root hydraulic conductivity, and hence replicates of the different genotypes were assessed at different times of day. In control conditions, the profligate genotypes (ICC 867 and ICC 14799) had significantly higher root hydraulic conductivity than the conservative genotypes (ICC 4958 and ICC 8058) ($P < 0.01$) (Fig. 3A).

The detached roots treated with 100 μM HgCl_2 inhibitor showed a dramatic decrease in root hydraulic conductivity but there were no significant differences between the profligate and conservative genotypes after HgCl_2 treatment (Fig. 3A). However, there was a significant interaction between the inhibitor effect and the category of genotype (profligate, conservative) (Table S2) in terms of the size of the decrease in root hydraulic conductivity following the treatment. Indeed, the profligate

genotypes ICC 867 (83%) and ICC 14799 (78%) had a larger reduction in root hydraulic conductivity than the conservative genotypes (68% in ICC 4958 and 70% in ICC 8058) (Fig. 3B) ($P < 0.05$).

Root anatomical differences

After apoplastic inhibition, chickpea root anatomy was examined under a microscope to assess the presence of $\text{Cu}_2[\text{Fe}(\text{CN})_6]$ precipitates. The chickpea root cylinder is composed of an epidermis consisting of elliptical cells surrounding a large cortex of ovoid parenchyma cells with intercellular spaces. The inner side of the cortex was delimited by an endodermis surrounding the vascular bundles (Fig. 4A). The endodermal cells were smaller than the cortical cells and were thickened on the inner tangential wall. The vascular bundles consisted of xylem (protoxylem and metaxylem vessels) in the centre and phloem at a peripheral position in the stele. Blockage of the apoplastic pathway with a combination of $\text{K}_4[\text{Fe}(\text{CN})_6]$ and CuSO_4 resulted in the whole root system appearing brown after treatment (Fig. 4B). The intercellular spaces also became progressively blocked with the insoluble crystals over time after treatment (Fig. 4C–E). In contrast, there was no sedimentation inside the cells.

Transpiration response to apoplast bypass flow inhibition with precipitates of CuSO_4

Transpiration was measured at 3.1 kPa (high VPD conditions) in all tested genotypes for almost 3 h prior to apoplast blockage treatment. Blockage of the apoplastic pathway (apoplastic pore) resulted in a rapid decrease in transpiration of all treated

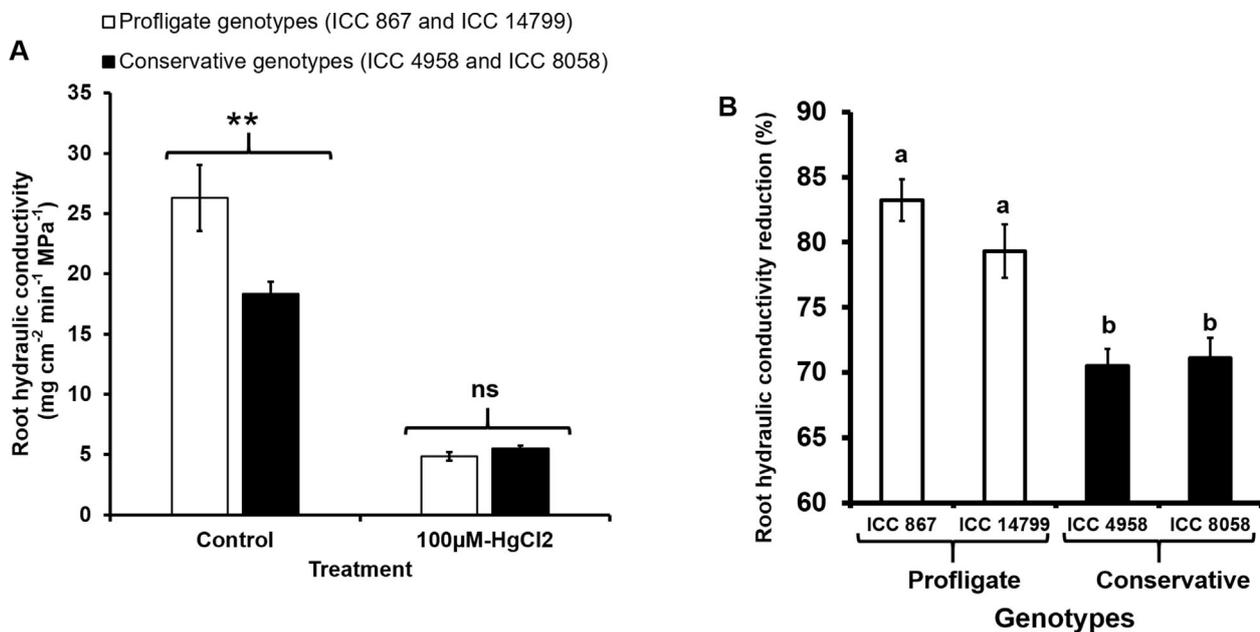


Fig. 3. Root hydraulic conductivity ($\text{mg}\cdot\text{H}_2\text{O}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}\cdot\text{MPa}^{-1}$) of detached roots (A) and percentage (%) inhibition of root hydraulic conductivity upon inhibitor treatment compared to controlled conditions (B). This was measured in four chickpea genotypes with contrasting plant transpiration response to VPD – profligate genotypes (ICC 867 and ICC 14799) and conservative genotypes (ICC 4958 and ICC 8058) – after the application of an aquaporin inhibitor (100 μM HgCl_2). ** $P < 0.01$, ns no significant difference between conservative and profligate genotypes in both control and 100 μM HgCl_2 conditions. Bars with different letters are significantly different ($P < 0.05$).

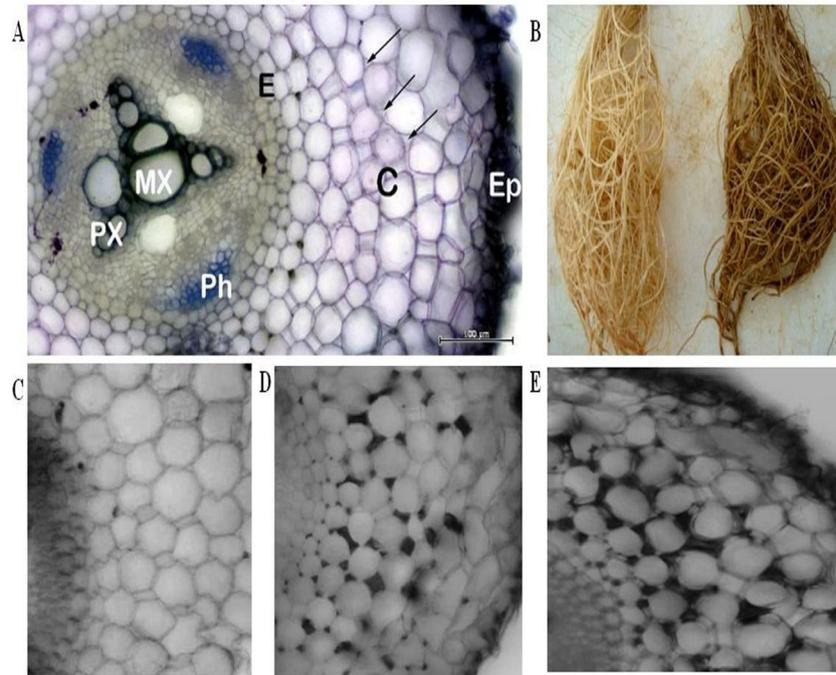


Fig. 4. Free-hand root cross-sections (A, C, D and E) and images (B) of chickpea after apoplast inhibition. Cross-section was made approximately 40 mm from root apex. Bars = 100 µm. (A) Over-view of root cylinder (epidermis – Ep, cortex – C, endodermis – E, phloem – Ph, protoxylem – PX, metaxylem – MX), with arrows to apoplastic pores. (B) Untreated (white, left) and treated (brown, right) chickpea roots after 3 h of apoplastic inhibition with CuSO_4 in plants earlier treated with 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$. C, D and E represent evolution precipitation of $\text{Cu}_2[\text{Fe}(\text{CN})_6]$ or $\text{Cu}[\text{CuFe}(\text{CN})_6]$ crystals 0, 1 and 3 h after CuSO_4 treatment.

plants about 20 min after CuSO_4 application (Fig. 5). The NTRR following apoplastic blockage was slightly but significantly lower in the conservative (40%) than in the profligate genotypes (50%) ($P < 0.001$), indicating that transpiration in conservative genotypes was more affected by apoplastic blockage than in profligate genotypes (Fig. S3).

DISCUSSION

Is restricted transpiration linked to the water transport pathway and root hydraulic conductivity?

The results of aquaporin-specific inhibition studies suggest that profligate chickpea genotypes depended more on the aquaporin-mediated water transport pathway than the conservative genotypes. These results are in agreement with similar results for other crop species, such as pearl millet (Tharanya *et al.*, 2017), sorghum (Choudhary *et al.*, 2013), wheat (Schoppach *et al.*, 2014), soybean (Sadok & Sinclair, 2010) and groundnut (Devi *et al.*, 2012), where conservative genotypes suffered less aquaporin inhibition than profligate genotypes. Conversely, results of the apoplast blockage experiments suggest that the conservative genotypes were more dependent on the apoplastic pathway than the profligate genotypes under high VPD conditions. This result is well supported with earlier results for pearl millet apoplastic inhibition, which had a higher decline in transpiration following inhibition of the apoplastic pathway in conservative genotypes than in profligate genotypes (Tharanya *et al.*, 2018). While there were clear differences in the water transport pathway between the conservative and profligate genotypes, the link between transport pathways and root

hydraulic conductivity was such that the conservative genotypes had lower root hydraulic conductivity than the profligate genotypes. Additional studies are needed to confirm this link. Nevertheless, these results, together with an earlier report, suggest that genotypes, across several species, having conservative plant water use would mostly rely on the apoplastic pathway for water transport in the roots. This may represent an advantage under scenarios where water is limited and needs to be retained in the soil profile, as is the case in environments where chickpea is grown. However, this particular feature may be less advantageous in scenarios where water is abundant and would require adjustments to channel water flow, as used by the profligate genotypes because of their higher dependence on the cell-to-cell pathway for water transport. Our hypothesis, that genotypes differing in the TR response to high VPD would also differ in their water transport pathway, was confirmed here in our and previous results, and provides an opportunity to target these specific genotype behaviours into breeding for specific water availability scenarios.

Is restricted transpiration under high VPD related to differences in plant vigour?

Restricted transpiration under high VPD might be linked to high plant vigour (rapid early development of leaf area and aboveground biomass). Indeed, a larger canopy would, intuitively, require more water to support transpiration, which may limit in its capacity to channel water through the plant under high evaporative demand than genotypes with a smaller canopy area. Data from experiments described here and from other unpublished experiments (Table 1) showed that profligate

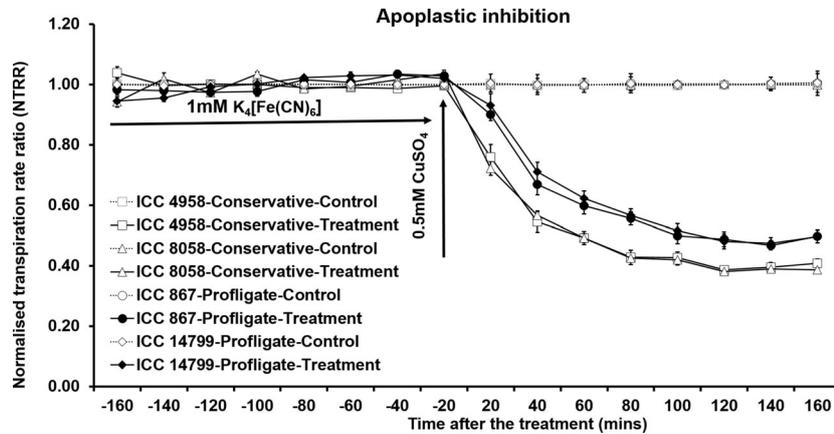


Fig. 5. Decline in normalized transpiration rate ratio (NTRR) following apoplast blockage, measured in four chickpea genotypes with contrasting transpiration response to VPD: ICC 867, ICC 14799 – profligate (filled circle and filled diamond) and ICC 4958, ICC 8058 – conservative (open square and open triangle). Each data point represents mean NTRR \pm SE of eight replicates per genotype. Apoplast pores were blocked by precipitation of $\text{Cu}_2[\text{Fe}(\text{CN})_6]$ or $\text{Cu}[\text{CuFe}(\text{CN})_6]$, following treatment with CuSO_4 of plants treated earlier with $\text{K}_4[\text{Fe}(\text{CN})_6]$. Plants were grown in hydroponics and tested at vegetative stage (26 days old).

genotypes had lower vigour, whereas conservative genotypes had higher vigour, confirming earlier findings (Zaman-Allah *et al.*, 2011a). Therefore, there appeared to be a link between the sensitivity of transpiration to increasing VPD and vigour. A similar interpretation was made in the case of pearl millet (Kholová *et al.*, 2010), where the conservative genotype also had a larger canopy. These results are also consistent with others for recombinant inbred lines of chickpea and their parents, where the conservative behaviour under high VPD was also linked to high vigour (Sivasakthi *et al.*, 2017). This link is important because, from a breeder's perspective, early vigour is an important trait for growth in water-limited environments (Richards, 2000; Botwright *et al.*, 2002; Richards & Lukacs, 2002), and here we show that the vigour trait may also be correlated with restricted transpiration, which would also lead to saving water. A question remains as to how high early vigour could be achieved in genotypes with conservative TR behaviour (and putatively restricted photosynthesis). Our interpretation is that these vigour differences occur early in the cropping cycle, when VPD is not high enough to trigger a restriction in transpiration. Early vigour may also contribute to higher yields because the shading of the soil surface by a large canopy would reduce incoming radiation and therefore limit evaporation from the soil, leaving more water available for the crop and increasing the crop's seasonal water use efficiency by as much as 25% (Siddique *et al.*, 1990). Of course, early vigour would also mean a faster use of the water in the soil profile and a higher risk of exposure to terminal stress conditions, especially for late-flowering genotypes (Zaman-Allah *et al.*, 2011b). Further work is required to test the trade-offs between traits contributing to crop water use, *i.e.* canopy characteristics, transpiration restriction, or any advantage from soil shading with a large canopy.

What is the link between water pathways, early vigour and drought tolerance?

Genotype ICC 4958 was considered tolerant because it flowers early, whereas ICC 8058 was considered sensitive because of the relatively late flowering. Both ICC 867 and ICC 14799 were considered tolerant to terminal drought despite their relatively

late flowering time (Krishnamurthy *et al.*, 2010; Zaman-Allah *et al.*, 2011b). The tolerance (ICC 4958, ICC 867 and ICC 14799) and sensitivity (ICC 8058) was shown to be a consequence of water saving mechanisms at an early stage, allowing these genotypes to retain more available soil water for the critical stages of reproduction and grain filling (Zaman-Allah *et al.*, 2011b). The sensitivity of ICC 8058 was seemingly related to its high early vigour combined with relatively late flowering and maturity, whereas the tolerance of ICC 4958 was a combination of early growth and water saving behaviour (and possible additional savings from lower soil water evaporation). These results suggest that water saving from restricted transpiration might be linked to a higher dependence on the apoplastic pathway for water transport in the root, which would only be beneficial for crops with specific phenology appropriate for a specific drought scenario. In other words, the water savings from restricted transpiration could not override the higher water demand of vigorous and late maturing genotypes like ICC 8058. In contrast, in genotypes that were less vigorous although profligate, like ICC 867 and ICC 14799 with low early vigour, even high transpiration under high VPD, putatively related to more dependence on the cell-to-cell water transport pathway, would be compensated by savings in water use. These interpretations reflect that fact that 'tolerance' to water stress is highly context-specific and follows a hierarchy of traits, where phenological duration, canopy development dynamics and capacity to restrict transpiration under high evaporative demand have important but interlinked roles. The role of stomatal control in this process is unclear. The above traits represent different scales of integration over time, with some traits changing within minutes (*e.g.* transpiration rate), while other traits are integrated over weeks (*e.g.* canopy development), thus one may interpret the role of stomata as being purely a response to the overall plant water balance.

CONCLUSION

In summary, a conservative transpiration response to increasing VPD was linked to a higher dependence on the apoplastic pathway for water transport and to high early vigour, whereas

profligate transpiration under high VPD was related to stronger involvement of the cell-to-cell pathway for water transport, higher root hydraulic conductivity and lower early vigour. Based on the knowledge of genotype performance under terminal water stress, it appeared that drought tolerance could be explained as a combination of three traits: restricted transpiration under high VPD, vigour and early flowering, with a positive relationship between the first two traits. Given that early flowering is the only trait currently used in breeding of drought tolerance in chickpea, this work opens new opportunities to design improved cultivars on the basis of their trait relationships.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure S1. Mean stabilized NTRR of profligate genotypes ICC 14799 and ICC 867 (filled bars) and conservative genotypes ICC 4958 and ICC 8058 (open bars) upon 1 mM H₂O₂ or 100 and 200 μM HgCl₂ inhibition. Data (n = 4) are mean values of stabilized NTRR after treatment. **P < 0.01, ***P < 0.001 are significant differences for conservative and profligate genotypes.

Figure S2. Root exudation rate (mg·H₂O·cm⁻²·min⁻¹) at different time points of four chickpea genotypes [ICC 4958, ICC 8058 (conservative) and ICC 867, ICC 14799 (profligate)]. Plants were grown in hydroponics and tested at vegetative stage (23 days old) using a pressure chamber. Root exudation was measured in detached roots at different time points (09:00–10:00 h, dashed line and filled diamond; 12:00–14:00 h, dotted lines with filled circle; and 17:00–18:00 h, solid lines with filled square) (A). Each data points represent mean root exudation rate (±SE) of five replicates per time points. (B) Slope of root exudation rate at specific time points: open bar 09:00–10:00 h, open bar with dashed lines 12:00–14:00 h, filled bar 17:00–18:00 h. Bars with different letters are significantly different (P < 0.05).

Figure S3. Mean stabilized NTTR values of profligate ICC 14799 and ICC 867 (filled bars) and conservative ICC 4958 and ICC 8058 (open bars) genotypes after apoplast blockage with Cu₂[Fe(CN)₆] or Cu[CuFe(CN)₆] crystals. Bars with different letters were significantly different (P < 0.05). ***P < 0.001.

Table S1. Details of vapour pressure deficit (VPD) stress imposition inside the growth chamber.

Table S2. Means with standard error and two-way ANOVA, and one-way ANOVA, on the results of root hydraulic conductivity under control and 100 μM-HgCl₂ inhibited conditions. Mean values followed by different alphabets indicate significant differences obtained through Tukey's test: L.S.D., Least significant difference.

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BLURB CONTENT

Profligate genotypes depend more on the cell-to-cell pathway, which could relate to their higher root hydraulic conductivity,

whereas conservative genotypes depend more on the apoplastic pathway which related to transpiration restriction under high VPD.