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24

Abstract

Water deficit influences leaf transpiration rate and photosynthetic activity. The genotype-dependent response of the latter has not been assessed in sunflower (*Helianthus annuus* L.), particularly during the reproductive period when grain filling and lipogenesis depend greatly on photosynthate availability. To evaluate genotypic responses to water deficit before and after flowering, two greenhouse experiments were performed. Four genotypes – two inbred lines (PSC8, XRQ) and two cultivars (Inedi, Melody) – were subjected to progressive water deficit. Non-linear regression was used to calculate the soil water deficit threshold (FTSWt) at which processes (transpiration and photosynthetic activity) were affected by water deficit. In the vegetative growth stage, photosynthetic activity was affected at a lower mean value of FTSWt (0.39) than transpiration (0.55). However, in the reproductive stage, photosynthetic activity was more sensitive to soil water deficit (FTSWt = 0.45). We found a significant ($p = 0.02$) effect of plant growth stage on the difference between photosynthesis and transpiration rate thresholds and, a significant ($p = 0.03$) effect of leaf age on transpiration. Such results will improve phenotyping methods and provide paths for integrating genotypic variability into crop models.

Keywords: genotype, net CO_2 assimilation rate, senescence, transpiration, water stress, *Helianthus annuus* L.

1. Introduction

Water deficit is a major factor limiting the cultivation of sunflower (*Helianthus annuus* L.) in southern Europe. Sunflower is cultivated during the summer, when evaporative demand is high, particularly during grain filling. In addition, sunflower is confined mostly to shallow soils, where water scarcity often occurs (Casadebaig, 2008; Terres Inovia, 2015). Even though sunflower is deemed tolerant to water deficits, it must be managed properly to optimize grain production and quality (Champolivier et al., 2011; Andrianasolo et al., 2016a; Andrianasolo et al.,2016b).

Patterns of water deficit response in sunflower are similar to those of most cultivated species (Hsiao, 1973; Chaves et al., 2002). They consist of early and progressive leaf stomatal closure due to high evaporative demand in the atmosphere and/or soil dryness and loss of leaf turgor. Stomata are likely regulated by abscisic acid and hydraulic signaling (Chaves et al., 2002; Pantin et al., 2012). At a moderate water deficit, photosynthetic activity decreases mainly due to stomatal closure. The decline in intercellular CO_2 following stomatal closure may induce a down-regulation of biochemical demand for carbon dioxide (Chaves et al., 2002). Connor and Hall (1997) reported that mechanisms involved in sunflower response to water stress varied with growth stage, suggesting that the control of plant water status through stomatal conductance changed with plant ontogeny (Pantin et al., 2012) before interacting with senescence processes. Since grain development and oil accumulation depend on available carbohydrates that originate mainly from photosynthetic activity after flowering (Merrien, 1992), any impairment of photosynthesis due to water deficit could likely reduce grain production and oil content. The sensitivity of photosynthesis to water deficit and its relationship to stomatal or non-stomatal limitations after flowering still needs to be investigated in sunflower.

Previous experiments have helped to highlight genetic variability in sunflower for photosynthetic processes and plant water status in response to water deficit (Maury et al., 1996; Maury et al., 2000; Kiani et al., 2007a; Kiani et al., 2007b). Genotypic differences were also found for transpiration response to water deficit (Casadebaig et al., 2008) in experiments in which plant water status was expressed as a fraction of transpirable soil water (FTSW). This method was established by Sinclair and Ludlow (1986), and later became routine for evaluating genotype and/or species response to progressive water deprivation (Liu et al., 2005; Pellegrino et al., 2006; Casadebaig et al., 2008; Verhoef and Egea, 2014). From the comparison of 25 different breeding generation sunflower genotypes, Casadebaig et al. (2008) distinguished two categories of responses to water deficit. The first, called the "conservative" strategy, consisted of an "early" stomatal closure at moderate water deficit (*i.e.* when the fraction of transpirable soil water is still high), leading to water conservation at the expense of photosynthesis. A second strategy, referred to as "productive", was characterized by a "later" stomatal closure, thereby allowing it to maintain prolonged photosynthetic activity (other things being equal). The delay between the start of stomatal closure (transpiration response) and its influence on photosynthesis is decisive for the potential productivity of a given genotype. In the SUNFLO crop model, Casadebaig et al. (2011) assumed that photosynthetic activity was influenced by water deficit after transpiration rate, irrespective of the plant growth stage and the genotype; they used an offset parameter value to distinguish the processes. We investigated whether this delay between transpiration and photosynthetic activity varies with genotype and/or plant growth stage.

Leaf developmental stages – or ages – involved in vegetative and reproductive periods differ in their functioning, growth history, microclimate environment, carbon

metabolism (Danuso et al., 1988) and senescence (Agüera et al., 2012). For example, young leaves can still undergo several stages of cell expansion and/or division, while fully expanded leaves reach their highest photosynthetic rates (Pantin et al., 2012; Nooden et al., 2012). In mature senescing leaves, aging progressively leads to accumulation of soluble sugars, a decrease in photosynthesis, and degradation of chlorophyll and the photosynthetic system triggered by oxidative stress (Aguëra et al., 2012). Sensitivity of these leaf types to water deficit can also differ; it was demonstrated that young leaves accumulated more proline than mature leaves when exposed to water deficit, and stomatal conductance and photosynthetic rates were more impaired in mature leaves (Cechin et al., 2006; Cechin et al., 2010). Yegappan et al. (1982) argued that impact of water deficit depended on the time of leaf life at which the stress occurred and on the intensity of the stress: mild stress affected unfolding leaves, while those still unfolded and expanding were only sensitive to severe stress. We did not assess differences in transpiration-rate response to water deficit between leaf ages. Different effects of plant growth stage on transpiration may be explained by differences in leaf developmental stages and in their regulation of stomatal conductance.

The aims of this study were to (i) analyze the response of transpiration and photosynthetic activity to water deficit in vegetative and reproductive stages in contrasting sunflower genotypes and (ii) evaluate the effect of leaf age on transpiration.

2. Materials and methods

2.1. Experimental design

Two greenhouse experiments were performed at the Institut National de la Recherche Agronomique (INRA) station in Auzeville, France (43°31'41.8" N, 1°29'58.6" E) in 2009 (Exp.I) and 2012 (Exp.II). In Exp.I, two inbred lines were used (XRQ and PSC8); their contrasting behaviors under water deficit had been previously determined (Rengel et al., 2012). In Exp.II, XRQ and two commercial F1 hybrids (Inedi and Melody) were used. Only XRQ was present in both experiments. Seeds were germinated in Petri dishes, and plantlets were rapidly transferred to large 15 L individual pots filled with a mixture of 50% clay loam, 40% P.A.M.2 potting soil (Proveen, distributed by Soprimex, Chateaurenard, Bouches-du-Rhône, France) and 10% sand. Seeds were sown on 1 April in 2009; two sowing dates were set in the 2012 experiment to obtain similar environmental conditions to simultaneously monitor vegetative and reproductive growth stages: 24 April and 16 March, respectively. Pots were randomly distributed within the greenhouse, and replicates were grouped into several blocks. There were 5 (2009) and 6 blocks/replicates (2012) and a total of 20 and 72 pots in 2009 and 2012, respectively. Plants were adequately irrigated and fertilized (Rengel et al., 2012; Marchand et al., 2013) in both experiments before water deficit was begun. Relative humidity and temperature of the air inside the greenhouse were recorded using thermo-hygrometers (ROTRONIC MP100A Temperature and Relative Humidity Probe, Campbell Scientific Ltd., Campbell Park, UK). Evaporative demand was estimated by calculating the vapor pressure deficit (VPD) according to Tetens (1930). Global radiation above the greenhouse was also monitored (CE-180, Cimel, France). Mean hourly photosynthetically active radiation was 72.4 and 114.0 J cm^{-2} in Exp.I and Exp.II, respectively.

2.1.1. Water deficit treatment

After reaching the 8-leaf stage (as for Casadebaig et al. (2008) and Marchand et al. (2013)) in the Exp.I and Exp.II "vegetative growth stage" experiment and the full flowering stage (R5.5, Schneiter and Miller, 1981) in the Exp.II "reproductive growth stage" experiment, we paired pots into non-irrigated/irrigated treatments. All pots were irrigated to full soil water saturation capacity the day before the water deficit experiment; no more water was provided to non-irrigated plants until the end of the experiment. Irrigated pots were re-watered daily to full soil water saturation capacity (200-700 ml, depending on daily evaporative demand and water consumption). All pots were covered with a 3 mm layer of polystyrene sheets to prevent soil evaporation. Soil evaporation was accounted for and estimated according to Marchand et al. (2013).

2.1.2. Measurements

2.1.2.1. Leaf transpiration

Transpiration rate at single-leaf level (TL, mmol H_2O m^{-2} s^{-1}) was measured within 2-3 min after closing the clamp of a porometer (LI-1600, LI-COR Inc., Lincoln, NE, USA) in Exp.I and a portable gas-exchange system (LI-COR 6400, Lincoln, Nebraska, NE, USA) in Exp.II. VPD was 1.9 ± 0.2 (mean ± standard deviation) in Exp.I and 1.2 ± 0.1 kPa in Exp.II. Temperatures were 26.8 ± 1.1°C in Exp.I and 25.5 ± 0.4°C in Exp.II. PPFD was higher than 800 µmol m^{-2} s^{-1} (natural sunlight) in Exp.I and ~1500 µmol m^{-2} s^{-1} (LI-COR 6400-02 light source, Lincoln, Nebraska, NE, USA) in Exp.II.

Transpiration rate was monitored daily on a fully expanded reference leaf (number 9 to 11 from the bottom of the plant) from 10 a.m. to 2 p.m. in the Exp.I and II vegetative growth stage. This leaf developmental stage is henceforth called "mature"

and corresponds to a dark green leaf, assumed to be experiencing its highest photosynthetic rate and having recently reached its maximum size; a leaf was considered "mature" at ~600°C-days from leaf initiation (Dosio et al,. 2003). In the reproductive growth stage (Exp.II), the chosen "mature" leaf laid in the upper one-third of the canopy (leaf number 18 to 22 from the bottom of the plant), assuming that this upper part of the canopy mostly contributes to total plant carbon assimilation (Alkio et al., 2003): its mean age was ~900°C-days from leaf initiation at the start of the experiment. Two other leaf nodes/ages were considered in Exp.I. One was a fully expanded aging leaf (called "post-maturing") that was "mature" at the start of experiment and reached the post-maturing phase during the experiment. The other, "young", corresponded to an expanding green leaf. The "mature" leaf node was selected as a function of plant growth to obtain similar thermal ages, and the "young" leaf always lays 3 nodes above the "mature" one. "Post-maturing" and "young" leaves were a mean of ~700 and ~530 °C-days old from initiation, respectively. Degree-day values of leaf initiation from plant emergence were estimated according to Dosio et al. (2003). A summary table of leaf ages is provided in Table 1. For further comparison of transpiration response to water deficit between growth stages and genotypes, we calculated a normalized indicator of leaf transpiration (Normalized Transpiration rate at single-Leaf level, NTL), which corresponded to the ratio of transpiration values between non-irrigated and irrigated plants.

Table.1

2.1.2.2. Leaf net photosynthesis

Leaf net photosynthesis rate (PA, µmol CO_2 m^{-2} s^{-1}) of the reference "mature" leaf was measured with the LI-COR 6400 device (Exp.II). Measurements were performed

under a saturated PPFD (~1500 µmol m^{-2} s^{-1}). Mean temperature and relative humidity during photosynthetic activity measurements were 25.5 ± 0.4°C and 40 ± 4%, respectively. Carbon dioxide concentration was 400 µmol mol^{-1}. As done for leaf transpiration, leaf net photosynthesis was converted into a normalized ratio between net photosynthetic activity of non-irrigated and irrigated plants (Normalized Photosynthetic Activity rate at single-leaf level, NPA).

2.1.2.3. Daily plant transpiration

Plant transpiration was estimated from daily measurements of pot weight and leaf area, following the method of Casadebaig et al. (2008). Daily weighings occurred at 5 p.m. and lasted 30 minutes. When a leaf displayed more than 50% senescence (yellowing or browning), its area was discarded from the daily leaf area measurement. Whole-plant transpiration rate (TP, kg m^{-2} day^{-1}) was obtained by dividing daily water loss by plant leaf area. Plant transpiration values were normalized (Normalized Transpiration rate at whole-Plant level, NTP) as the ratio between non-irrigated and irrigated values for further comparison of water use dynamics.

2.1.2.4. Water deficit experienced by the plant

FTSW (fraction of transpirable soil water) was used as an indicator of water deficit experienced by the plant (Sinclair, 2005). It was calculated from daily non-irrigated pot weight ($pot\ weight\ j$), pot weight at saturation water capacity ($pot\ weight\ sc$) and pot weight when leaf transpiration of the non-irrigated pot was less than 10% of its corresponding control pair (NTL < 10%; $pot\ weight\ 10\%$), such that:

$$FTSW = \frac{(pot\ weight\ j - pot\ weight\ 10\%)}{(pot\ weight\ sc - pot\ weight\ 10\%)} \quad eq(1)$$

Since calculation of FTSW considers transpiring leaf surfaces, such standardization enables comparison of genotypes that differ in leaf area and/or levels of transpiration rate per unit leaf area.

2.2. Modeling the response of transpiration and photosynthesis to soil water deficit and statistical analysis

Dynamics of transpiration and photosynthesis response to FTSW were adjusted with a modified version of the Casadebaig et al. (2008) model:

$$y = \frac{1}{1 + 4.5 \times exp(a \times FTSW)} \quad eq(2)$$

where y corresponds to the physiological process (*i.e.* NTL, NPA or NTP) and a to the model parameter describing the shape of the response of the physiological process to FTSW. As a increases, the process modeled starts to decrease at a higher FTSW. Fits were performed with R software v. 3.0.2 (R Core Team, 2014) using *nls* regression. Quality of fit (root mean squared error (RMSE) and R^2) was assessed. Values of a were compared between genotypes, leaf ages and plant growth stages using ANOVA. Fisher's Least Significant Difference (LSD) test was used to determine groups when effects of genotype, plant growth stage or leaf age were significant. Corresponding FTSWt values, *i.e.* FTSW values at which transpiration or photosynthesis starts to decrease, were calculated from eq (2), assuming that FTSWt was achieved when maximum normalized variables were reduced by 0.25%; this threshold was chosen to estimate the time at which processes begin to be influenced by water deficit. Higher FTSWt values are interpreted as higher "sensitivity" of a given process to water deficit.

Values of leaf transpiration, plant transpiration and photosynthetic rates were compared by experiment and/or growth period and/or leaf developmental stage with ANOVA; we established LSD-based groups when the difference was significant. Correlations between transpiration rates at the single-leaf (NTL) and whole-plant (NTP) levels were calculated and then assessed with the Student's t-test using R.

3. Results

3.1. Comparison of single-leaf and whole-plant transpiration rates and photosynthetic activity

Transpiration rates of mature leaves of XRQ at vegetative growth stage (TL, Table.2) were higher for irrigated plants in Exp.I than in Exp.II (12.90 vs. 5.94 mmol m^{-2} s^{-1}, respectively). Transpiration rates at whole-plant level (TP) were also higher in Exp.I than in Exp.II. Photosynthetic activity rates (PA) ranged from 1.28 to 15.85 µmol CO_2 m^{-2} s^{-1} depending on the water status and growth stage. For the three processes (PA, TL, TP), values for non-irrigated plants were always significantly lower than those for irrigated plants.

Table.2

3.2. Responses of single-leaf transpiration and photosynthesis to soil water deficit in vegetative and reproductive stages

NTL and NPA of Inedi, Melody and XRQ genotypes were compared at the single-leaf level ("mature" leaves) in vegetative and reproductive periods. Values of a for NTL (a.NTL) tended to be lower in the reproductive stage than in the vegetative stage (Table.3). Growth-stage effect on a.NTL was significant (mean = -13.74 for vegetative and -17.91 for reproductive growth stages, $p<0.01$). Neither genotype nor

growth stage effects in a.NPA were detected (mean = -18.76), but the effect of their interaction (genotype × growth stage) was significant ($p<0.01$). When calculating the difference between a for NTL and NPA, we detected significant growth-stage ($p<0.01$) and growth-stage × genotype effects ($p<0.05$) (Table.4). In the vegetative period, NTL began decreasing earlier than NPA, and a delay occurred between them (NPA had lower FTSWt than NTL, Table.3). In the reproductive period, a.NTL-a.NPA was positive for Melody (Table.5), suggesting that in this genotype transpiration rate was decreased before photosynthetic activity at the single-leaf level. For the two other genotypes, with negative differences, transpiration rate was decreased after photosynthetic activity at the single-leaf level (Table.3). Mean a.NTL- a.NPA values displayed higher variability in the reproductive period (Tables.4 and 5). FTSW thresholds for transpiration rate were generally higher at the single-leaf level (NTL) than at the whole-plant level (NTP).

Fig.1

Table.3

Table.4

Table.5

3.3. Response of transpiration rate at the single-leaf level to soil water deficit depends on leaf age

When comparing NTL of 3 leaf ages of 2 genotypes (XRQ and PSC8) in Exp.I, leaf age and genotype had significant effects ($p<0.01$ and $p<0.05$, respectively) on a.NTL (Fig.2 and Table.6). In both genotypes, “post-maturing” and “young” leaf transpiration rate decreased at a similar FTSWt (mean ≈ 0.78). Transpiration rate

response to soil water deficit of "mature" leaves was less sensitive (mean FTSWt = 0.24 and 0.46 for PSC8 and XRQ, respectively; Table.7), and the lower sensitivity of PSC8 contributed to significant genotypic differences in a.NTL.

Fig.2

Table.6

Table.7

3.4. Correlations between transpiration rates at single-leaf and whole-plant levels

When investigating the relationship between NTL and NTP in Exp.I, NTL was significantly correlated with NTP for all leaf ages (Fig.3). "Post-maturing" and "mature" leaves had the strongest relationships (R^2 = 0.67 and 0.68, respectively). R^2 was on average lower in Exp.I (0.65) than in Exp.II (0.83); in the latter, NTP and NTL had a slightly stronger relationship in the vegetative growth stage (Fig.4).

Fig.3

Fig.4

4. Discussion

Methodological limitations and potential interactions with the growth environment

Comparison of VPD in the two experiments showed that evaporative demand was greater in Exp.I than in Exp.II, mainly due to higher temperatures in the former. Greater evaporative demand generally increases the sensitivity thresholds of stomatal conductance to soil water deficit (Sadras and Milroy 1996). Since the XRQ genotype was present in both experiments (vegetative growth stage), we investigated the influence of water deficit on leaf transpiration rate at the single-leaf level. NTL

began to decrease at higher FTSWt in Exp.I (mean FTSWt = 0.67) than in Exp.II (FTSWt = 0.63). However, compared to the genotype PSC8 that was planted the same year (2012) in another experiment (data not shown), we observed the same genotype classification as Casadebaig et al. (2008): leaf transpiration began to decrease at higher FTSWt for XRQ than PSC8. The small number of genotypes that we studied is compensated by the fact that two of them (XRQ and PSC8) have contrasting behaviors in response to water deficit (Rengel et al., 2012). The range of FTSWt for NTL of the 4 genotypes studied here is as large as that of the 25 genotypes studied by Casadebaig et al. (2008).

Leaves differ in sensitivity to water deficit by growth stage, and distribution of leaf developmental stages explains differences in pre- and post-flowering plant transpiration

Our results showed that the influence of water deficit on transpiration rates differed depending on the age of leaves: “mature” leaves were the least sensitive to water deficit. This is in line with Pantin et al. (2012), who argued that stomatal regulation progressively appeared as leaves aged; in our case, “young” leaves might have displayed more sensitivity to environmental conditions that do not involve stomatal closure. “Post-maturing” leaves are probably becoming senescing leaves that can no longer control water loss through stomata. It may be surprising that the age of a “mature” leaf differs between vegetative and reproductive stages (600 and 900°C-days, respectively). Moschen et al. (2014) showed that leaf profile and senescence varied with developmental stage; higher leaves are initiated later than lower leaves but live longer. We assumed that “mature” leaves in vegetative and reproductive periods could be considered similar, at least in terms of maximum photosynthetic capacity.

The differences observed in leaf functioning in vegetative and reproductive periods were linked to the distribution of leaf developmental stages, which vary with plant growth stage. At a given growth stage, the leaf population is a mixture of “young”, “mature”, and “post-maturing” leaves. At the start of the vegetative growth stage experiment their percentages were 33%/33%/33%, respectively, while at the start of the reproductive growth stage experiment they were 20%/60%/20%, respectively. More “mature” leaves were observed during the latter stage because maximum leaf expansion is reached at mid-flowering (Merrien, 1992). The later influence of water deficit on transpiration after flowering (*i.e.* lower FTSW threshold for NTP) is explained by the higher percentage of “mature” leaves, which were less sensitive than other leaf developmental stages. This finding does not agree with the conclusions of Connor and Hall (1997), who reported that stomatal conductance was more sensitive to water deficit in the reproductive growth stage than in the vegetative growth stage. This lower sensitivity of plant transpiration rate to water deficit was observed at a daily scale in our data, but it could be linked to differences in biomass and consequent water uses post-flowering. This would preclude comparison between the two experiments; by using FTSW, we demonstrated that the transpiration rate of plants in post-flowering periods has less sensitivity to water deficit than in pre-flowering periods.

Photosynthetic activity response to water deficit in vegetative and reproductive growth stages

In the vegetative growth stage, leaf transpiration was influenced by water deficit at a higher FTSWt than leaf photosynthesis. This delay did not exist or was reduced in the reproductive period, with inversions occurring between transpiration and photosynthesis thresholds. This suggests non-stomatal limitation of photosynthesis in

response to post-flowering water deficit. However, the Melody genotype appeared capable of maintaining photosynthesis at lower FTSWt than the other genotypes. Kiani et al. (2007a) showed that down-regulation of fructose 1,6-bisphosphatase could play a role in non-stomatal limitation of photosynthesis, decreasing photosynthesis under water deficit. Key genes associated with leaf transpiration rate and water plant status whose expression differs in sensitive and tolerant genotypes were also identified in sunflower (Rengel et al., 2012). Exp.II enabled gene expression in leaves to be monitored during water deficit, which increased understanding of the physiological basis of genetic variability in sunflower response to water deficit. The latter helped in developing a biomarker for plant water status in sunflower (Marchand et al., 2013). The existing delay between the responses of transpiration and photosynthesis at single-leaf level (NTL and NPA) to increasing water deficit can now be more accurately predicted by the SUNFLO crop model (Casadebaig et al., 2011).

Existing genotypic differences requiring further investigation

A genotype effect was observed for transpiration response at the single-leaf level in Exp.I; “mature” leaves of PSC8 maintained transpiration at a significantly lower fraction of transpirable soil water than XRQ. Genotype effects were not significant for individual processes (NTL and NPA) in Exp.II because genotypes did not differ enough (differences of 0.17 and 0.22 in mean FTSWt in Exp.II and Exp.I, respectively). Genotype effect was observed in the delay between transpiration and photosynthesis in response to increasing water deficit, particularly in the vegetative period. While other experiments have studied the response of transpiration to water deficit during the vegetative growth stage (Casadebaig et al. 2008), our results for the reproductive growth stage are new and require support from future experiments for a

wider range of genotypes. Monitoring water deficit in post-flowering plants remains difficult because the latter are too large for the standard pots used in greenhouse experiments. As suggested by Adiredjo et al. (2014), leaf carbon-isotope discrimination should be performed to assess variability in genotypic water use efficiency. Comparing leaf and plant transpiration responses to water deficit confirmed that choosing a "mature" leaf as a reference (Cechin et al., 2006; Casadebaig et al., 2008; Cechin et al., 2010) was relevant for high-throughput varietal assessment regardless of growth stage. However, leaf and plant transpiration rates were not measured at the same time step; to confirm our observations, both single-leaf and whole-plant transpirations should be measured over identical 24-hour periods.

5. Conclusions

This study analyzed responses of transpiration and photosynthesis of sunflower genotypes to soil water deficit as a function of growth stage (before and after flowering) and leaf age. We demonstrated that transpiration was influenced by water deficit before photosynthesis during the vegetative period, while no significant delay occurred between processes in the reproductive growth stage. Our results generate pathways for improving phenotyping methods under water deficit and exploring genetic variability in sunflower. Our results suggest that including the sensitivity of both processes to water deficit as a function of growth stage in the SUNFLO crop model should help to predict sunflower response to a wider range of soil water deficits.

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407 **7. References**

408 Adiredjo AL, Navaud O, Lamaze T, Grieu P (2014) Leaf carbon isotope discrimination as an
409 accurate indicator of water-use efficiency in sunflower genotypes subjected to five stable
410 soil water contents. Journal of Agronomy and Crop Science 200, 416–424.

411 Andrianasolo FN, Champolivier L, Debaeke P, Maury P (2016a) Source and sink indicators
412 for determining nitrogen, plant density and genotype effects on oil and protein contents in
413 sunflower achenes. Field Crops Research (in press)

414 Andrianasolo FN, Debaeke P, Champolivier L, Maury P (2016b) Analysis and modelling of
415 the factors controlling seed oil concentration in sunflower: a review. Oléagineux, Corps gras,
416 Lipides 23, D206, doi: 10.1051/ocl/2016004.

417 Agüera E, Cabello P, de la Mata L, Molina E, de la Haba P (2012) Metabolic regulation of
418 leaf senescence in sunflower (*Helianthus annuus* L.) plants. In: Senescence (T. Nagata, ed.),
419 InTech Open Access Publisher, doi: 10.5772/33671.

420 Alkio M, Schubert A, Diepenbrock W, Grimm E (2003) Effect of source–sink ratio on seed
421 set and filling in sunflower (*Helianthus annuus* L.). Plant, Cell & Environment 26, 1609–
422 1619.

Casadebaig P (2008) Analyse et modélisation dynamique des interactions génotype-environnement-conduite de culture: application au tournesol (*Helianthus annuus* L.). Doctoral thesis, INP Toulouse, France.

Casadebaig P, Debaeke P, Lecoeur J (2008) Thresholds for leaf expansion and transpiration response to soil water deficit in a range of sunflower genotypes. European Journal of Agronomy 28, 646–654.

Casadebaig P, Guilioni L, Lecoeur J, Christophe A, Champolivier L, Debaeke P (2011) SUNFLO, a model to simulate genotype-specific performance of the sunflower crop in contrasting environments. Agricultural and Forest Meteorology 151, 163–178.

Cechin I, Corniani N, Fumis T de F, Cataneo AC (2010) Differential responses between mature and young leaves of sunflower plants to oxidative stress caused by water deficit. Ciência Rural 40, 1290–1294.

Cechin I, Rossi SC, Oliveira VC, Fumis TF (2006) Photosynthetic responses and proline content of mature and young leaves of sunflower plants under water deficit. Photosynthetica 44, 143–146.

Champolivier L, Debaeke P, Merrien A (2011) Pourquoi irriguer le tournesol, une culture réputée tolérante à la sécheresse? Innovations Agronomiques-CIAG INRA 14, 151-164.

Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CPP, Osório ML, Carvalho I, Faria T, Pinheiro C (2002) How plants cope with water stress in the field? Photosynthesis and growth. Annals of Botany 89, 907–916.

Connor DJ, Hall AJ (1997) Sunflower physiology. In: Sunflower technology and production. Schneiter A.A. (ed), ASA, Madison, Wisconsin, USA, 113-182.

Danuso F, Vedove GD, Peressotii A (1988) Photosynthetic response of sunflower leaves to PPFD under field conditions, with relation to their age and position. In Proceedings of the XIIth International Sunflower Conference 1, 95–102

448 Dosio GA, Rey H, Lecoeur J, Izquierdo NG, Aguirrezábal LA, Tardieu F, Turc O (2003) A
449 whole-plant analysis of the dynamics of expansion of individual leaves of two sunflower
450 hybrids. Journal of Experimental Botany 54, 2541–2552.

451 Hsiao TC (1973) Plant responses to water stress. Annual Review of Plant Physiology 24,
452 519–570.

453 Kiani SP, Grieu P, Maury P, Hewezi T, Gentzbittel L, Sarrafi A (2007) Genetic variability
454 for physiological traits under drought conditions and differential expression of water stress-
455 associated genes in sunflower (*Helianthus annuus* L.). Theoretical and Applied Genetics
456 114, 193–207.

457 Kiani SP, Talia P, Maury P, Grieu P, Heinz R, Perrault A, Nishinakamasu V, Hopp E,
458 Gentzbittel L, Paniego N, Sarrafi A (2007) Genetic analysis of plant water status and
459 osmotic adjustment in recombinant inbred lines of sunflower under two water treatments.
460 Plant Science 172, 773–787.

461 Lawlor DW (2002) Limitation to Photosynthesis in Water-stressed Leaves: Stomata vs.
462 Metabolism and the Role of ATP. Annals of Botany 89, 871–885.

463 Liu F, Andersen MN, Jacobsen S-E, Jensen CR (2005) Stomatal control and water use
464 efficiency of soybean (*Glycine max* L. Merr.) during progressive soil drying. Environmental
465 and Experimental Botany 54, 33–40.

466 Marchand G, Mayjonade B, Varès D, Blanchet N, Boniface M-C, Maury P, Andrianasolo
467 FN, Burger P, Debaeke P, Casadebaig P, Vincourt P, Langlade N (2013) A biomarker based
468 on gene expression indicates plant water status in controlled and natural environments. Plant,
469 Cell & Environment 36, 2175–2189.

470 Maury P, Berger M, Mojayad F, Planchon C (2000) Leaf water characteristics and drought
471 acclimation in sunflower genotypes. Plant and Soil 223, 155–162.

Maury P, Mojayad F, Berger M, Planchon C (1996) Photochemical response to drought acclimation in two sunflower genotypes. Physiologia Plantarum 98, 57–66.

Moschen S, Bengoa Luoni S, Paniego NB, Hopp HE, Dosio GAA, Fernandez P, Heinz RA (2014) Identification of Candidate Genes Associated with Leaf Senescence in Cultivated Sunflower (*Helianthus annuus* L.) PLoS ONE 9, e104379. doi:10.1371/journal.pone.0104379.

Noodén LD (2012) 'Senescence and aging in plants.' (Elsevier Ed.), Academic Press 564 p.

Pantin F, Simonneau T, Muller B (2012) Coming of leaf age: control of growth by hydraulics and metabolics during leaf ontogeny. New Phytologist 196, 349–366.

Pellegrino A, Gozé E, Lebon E, Wery J (2006) A model-based diagnosis tool to evaluate the water stress experienced by grapevine in field sites. European Journal of Agronomy 25, 49–59.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Rengel D, Arribat S, Maury P, Martin-Magniette M-L, Hourlier T, Laporte M, Varès D, Carrère S, Grieu P, Balzergue S, Gouzy J, Vincourt P, Langlade N (2012) A gene-phenotype network based on genetic variability for drought responses reveals key physiological processes in controlled and natural environments. PLoS One, e45249, doi: 10.1371/journal.pone.0045249.

Sadras VO, Milroy SP (1996) Soil-water thresholds for the responses of leaf expansion and gas exchange: A review. Field Crops Research 47, 253–266.

Schneiter AA, Miller JF (1981) Description of sunflower growth stages. Crop Science 21, 901–903.

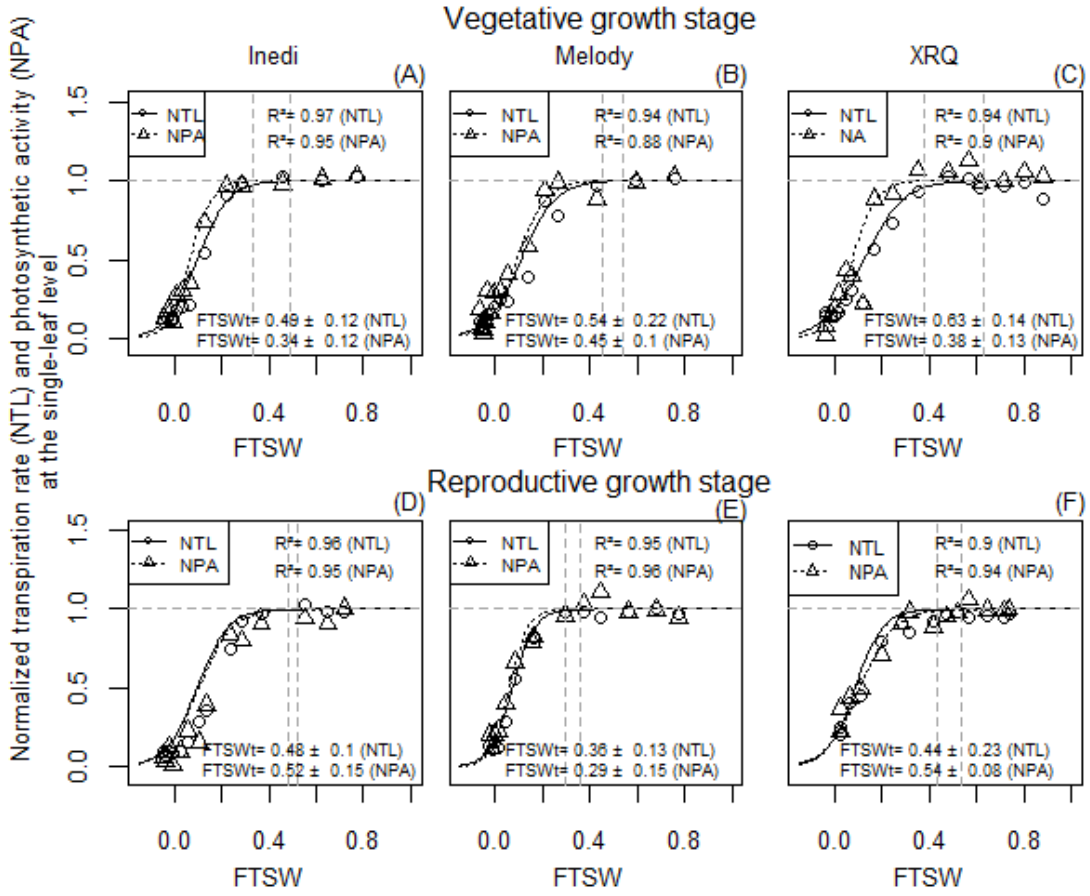
Sinclair TR (2005) Theoretical analysis of soil and plant traits influencing daily plant water flux on drying soils. Agronomy Journal 97, 1148–1152.

Sinclair TR, Ludlow MM (1986) Influence of soil water supply on the plant water balance of four tropical grain legumes. Australian Journal of Plant Physiology 13, 329–341.

Tetens, Otto (1930) Uber einige meteorologische Begriffe. Zeitschrift fur Geophysik 6, 297-309.

Terres Inovia, 2015. Institut technique des producteurs d'oléagineux, de protéagineux, de chanvre et de leurs filières. URL: www.terresinovia.fr (accessed 09 July 2015)

Yegappan TM, Paton DM, Gates CT, Müller WJ (1982) Water stress in sunflower (*Helianthus annuus* L.) 2. Effects on leaf cells and leaf area. Annals of Botany 49, 63–68.



505

506 Fig.1. Responses of normalized transpiration rate (NTL) and photosynthetic activity
507 (NPA) at the single-leaf level to fraction of transpirable soil water (FTSW) in (A, B,
508 C) vegetative and (D, E, F) reproductive growth stages for 3 genotypes (Inedi,
509 Melody, XRQ) during Exp.II. Quality of fit (R^2) and corresponding means ± standard
510 deviations of FTSWt threshold values are provided for each process. Vertical dashed
511 lines indicate FTSWt thresholds.

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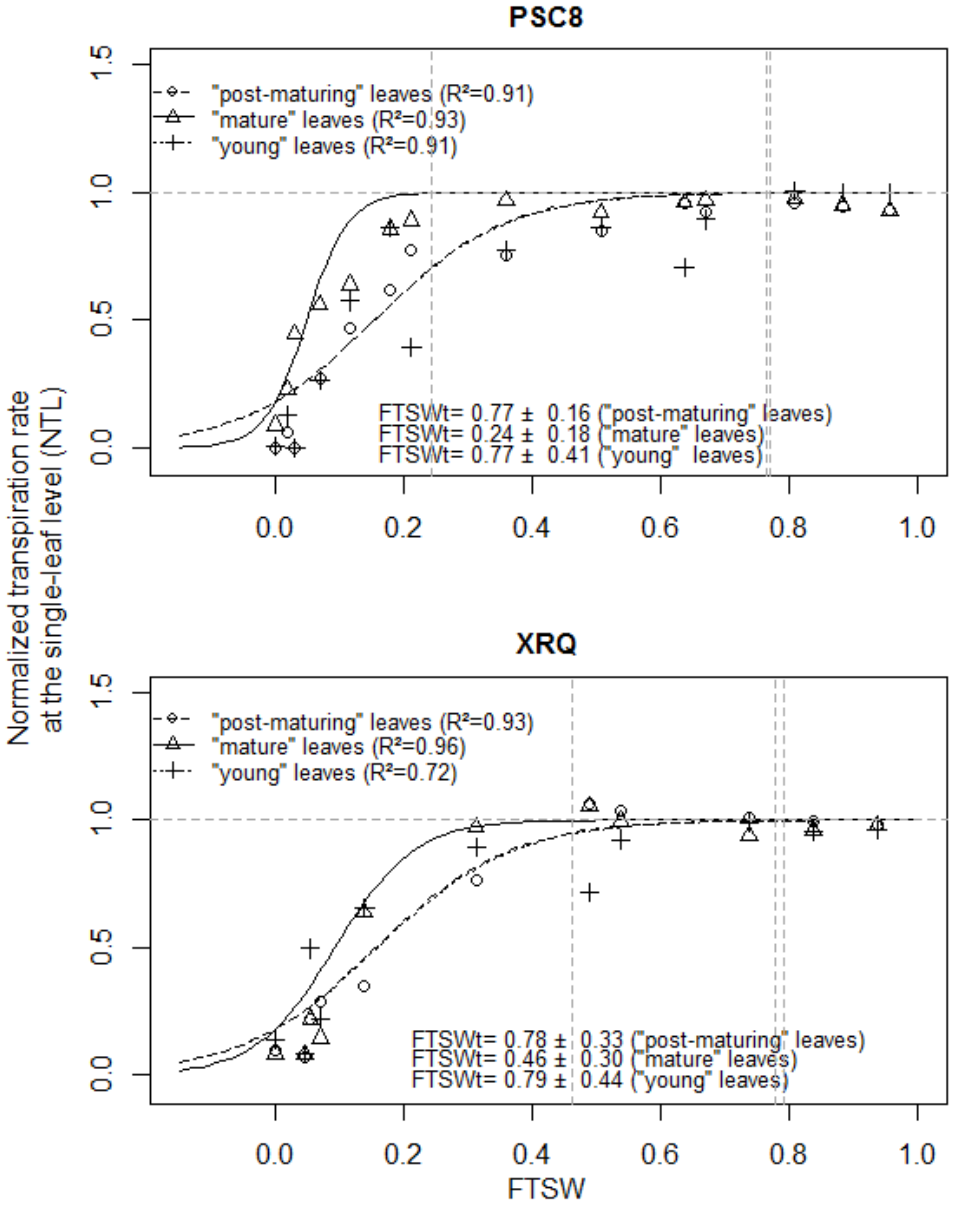


513

514 Fig.2. Responses of normalized transpiration rate at the single-leaf level (NTL) to
515 fraction of transpirable soil water (FTSW) in 2 genotypes (PSC8, XRQ) and 3 leaf
516 ages in Exp.I. Leaf ages were categorized into aging leaves ("post-maturing"),
517 recently fully expanded leaves ("mature") and young expanding leaves ("young").
518 Quality of fit (R^2) and corresponding means ± standard deviations of FTSWt
519 threshold are indicated. Note that fitted curves for "post-maturing" and "young"
520 leaves overlap. Vertical dashed lines indicate FTSWt thresholds.

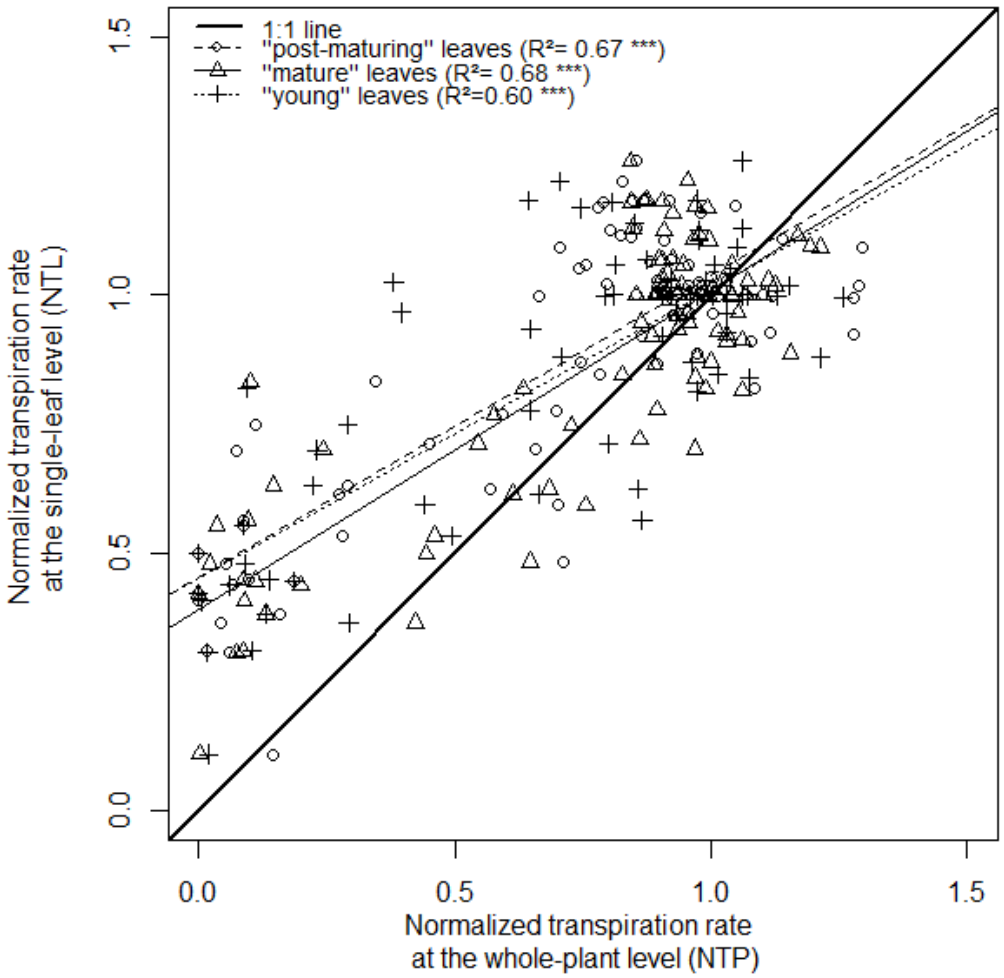
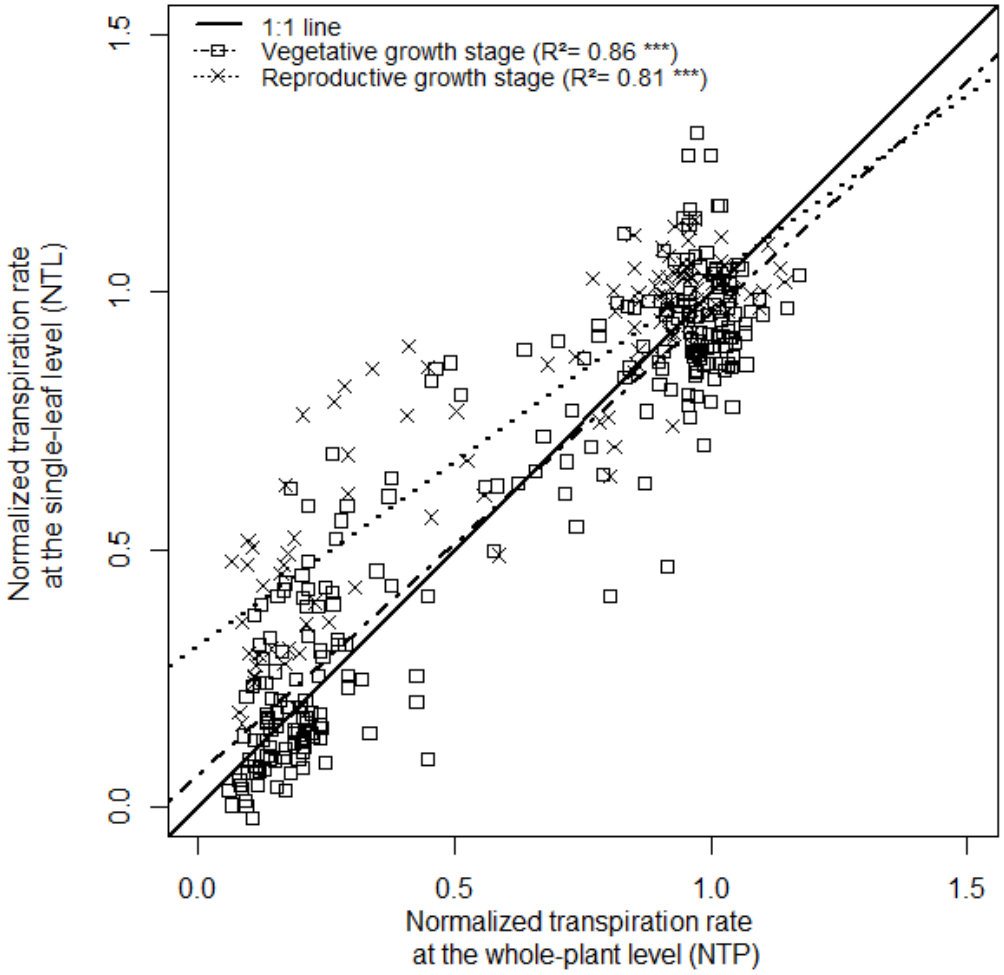


Fig.3. Relationship between normalized transpiration rates at single-leaf (NTL) and whole-plant (NTP) levels in Exp.I. Relationships were assessed by leaf age.



526

527 Fig.4. Relationship between normalized transpiration rates at single-leaf (NTL) and
528 whole-plant (NTP) levels in Exp.II. Relationships were assessed by plant growth
529 stage.

530

531 Table.1. Characteristics of leaves of different ages (°C-days from initiation) used (•)
532 in Exp.I and Exp.II. n = not used

Name	Definition	Growth stage	Age	Position from bottom	Exp.	
					I	II
"post-maturing"	mature leaf at the start of experiment, in post-expansion thereafter	Vegetative	700	9	•	n
"mature"	dark green leaf with its highest photosynthetic rate and having recently reached its maximum size	Vegetative	600	9 to 11	•	•
		Reproductive	900	18 to 22	n	•
"young"	green expanding leaf	Vegetative	530	12 to 14	•	n

533

534

535 Table.2. Means ± standard deviations of net photosynthetic activity (PA) and
536 transpiration rate at single-leaf (TL) and whole-plant (TP) levels for the genotype
537 XRQ two days before the end of Exp.I and Exp.II. In each line, letters indicate
538 groups (determined using Fisher's Least Significant Difference test) with significant
539 differences between irrigated and non-irrigated plants at $p<0.05$. Non-irrigated and
540 irrigated situations were distinguished as well as leaf age in Exp.I experiment and
541 growth stage in Exp.II. PA was not measured in Exp.I. n/a = not available

Plant growth stage	Leaf name	PA (μmol CO_2 m^{-2} s^{-1})		TL (mmol H_2O m^{-2} s^{-1})		TP (kg m^{-2} day^{-1})	
		Non-irrigated	Irrigated	Non-irrigated	Irrigated	Non-irrigated	Irrigated
Exp.I							
Vegetative	"Post-maturing"	n/a		2.40 ± 1.51 a	11.23 ± 2.20 b	4.40 ± 1.70 a	5.90 ± 0.40 b
	"Mature"			2.97 ± 2.78 a	12.90 ± 2.30 b		
	"Young"			3.80 ± 2.33 a	8.26 ± 1.62 a		
Exp.II							
Vegetative	"Mature"	1.28 ± 0.81 a	15.85 ± 2.28 b	0.81 ± 0.04 a	5.94 ± 0.80 b	0.13 ± 0.09 a	1.05 ± 0.03 b
Reproductive		4.25 ± 3.17 a	13.44 ± 2.74 b	1.99 ± 0.88 a	5.97 ± 0.75 b	0.58 ± 0.22 a	1.48 ± 0.17 b

542

543

544 Table.3. Summary table of response parameters of water deficit for normalized
545 photosynthetic activity (NPA), transpiration at the single-leaf level (NTL) and
546 transpiration at the whole-plant level (NTP). Fitted values of *a*, fraction of

547 transpirable soil water (FTSWt) values and indicators of quality of fit (root mean
548 squared error (RMSE) and R^2) are presented per genotype and growth stage for
549 Exp.II. The *a* parameter describes the shape of the response of the process to FTSW.

Plant growth stage	Genotype	Parameter	NPA	NTL	NTP
Vegetative	Inedi	*a*	-22.31	-15.31	-12.58
		FTSWt	0.34	0.49	0.60
		RMSE	0.11	0.09	0.11
		R^2	0.95	0.97	0.95
	Melody	*a*	-16.48	-13.93	-15.00
		FTSWt	0.45	0.54	0.50
		RMSE	0.16	0.10	0.11
		R^2	0.88	0.94	0.95
	XRQ	*a*	-19.82	-11.97	-19.02
		FTSWt	0.38	0.63	0.39
		RMSE	0.14	0.10	0.13
		R^2	0.90	0.94	0.89
Reproductive	Inedi	*a*	-14.35	-15.51	-17.98
		FTSWt	0.52	0.48	0.42
		RMSE	0.11	0.10	0.18
		R^2	0.95	0.96	0.91
	Melody	*a*	-25.60	-21.08	-25.96
		FTSWt	0.29	0.36	0.29
		RMSE	0.10	0.11	0.12
		R^2	0.96	0.95	0.88
	XRQ	*a*	-14.00	-17.14	-29.09
		FTSWt	0.54	0.44	0.26
		RMSE	0.11	0.09	0.12
		R^2	0.94	0.90	0.78

550

551

552 Table.4. ANOVA of the difference between response parameters of normalized
553 transpiration rate and photosynthetic activity at the single-leaf level (a.NTL- a.NPA)
554 in Exp.II. Asterisks indicate sources significant at $p<0.05$.

a .NTL-*a* .NPA Source of variation	df	Sum Sq	Mean Sq	F	p
Growth stage	1	264.6	264.7	6.61	0.02*
Genotype	2	8.4	4.2	0.11	0.90
Block	2	22.0	11.0	0.28	0.76
Growth stage x genotype	2	277.5	138.7	3.47	0.05*
Residuals	25	1000.6	40.0		

555

556

557 Table.5. Means ± standard deviations of differences in response parameters between
558 normalized transpiration rate and photosynthetic activity at the single-leaf level
559 (a.NTL- a.NPA) in Exp.I. Significant effects of genotype and growth stage were
560 tested with ANOVA. Means followed by common letter(s) are not significantly
561 different at $p<0.05$ by Fisher's Least Significant Difference test.

a .NTL-*a* .NPA	Growth stage	
Genotype	Vegetative	Reproductive
Inedi	7.00 ± 7.91 ab	-2.17 ± 7.20 bc
Melody	2.55 ± 2.50 abc	4.52 ± 5.99 ab
XRQ	7.85 ± 5.62 a	-3.14 ± 6.79 c

562

563

564 Table.6. ANOVA table of the values of a parameter for normalized transpiration rate
565 at the single-leaf level (NTL) in Exp.I. Asterisks indicate sources significant at
566 $p<0.05$.

a .NTL Source of variation	df	Sum Sq	Mean Sq	F	p
Genotype	1	625.8	625.8	6.41	0.02*
Leaf age	2	830.7	415.3	4.26	0.03*
Block	1	65.4	65.4	0.67	0.42
Genotype x leaf age	2	151.0	75.5	0.77	0.47
Residuals	23	2244.3	97.6		

567

568

Table.7. Summary table of response parameters to water deficit for normalized transpiration rate at single-leaf and whole-plant levels (NTL and NTP respectively) during Exp.I. Fitted values of a, fraction of transpirable soil water (FTSWt) values and indicators of quality of fit (root mean squared error (RMSE) and R^2) are presented per genotype and leaf age. The a parameter describes the shape of the response of the process to FTSW.

Growth stage	Genotype	Parameter	NTL			NTP
			"post-maturing" leaves	"mature" leaves	"young" leaves	
Vegetative	PSC8	*a*	-9.79	-31.01	-9.75	-24.65
		FTSWt	0.77	0.24	0.77	0.30
		RMSE	0.12	0.09	0.11	0.13
		R^2	0.91	0.93	0.91	0.86
	XRQ	*a*	-9.63	-16.26	-9.48	-28.48
		FTSWt	0.78	0.46	0.79	0.26
		RMSE	0.12	0.10	0.19	0.14
		R^2	0.93	0.96	0.72	0.81