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4 MANUSCRIPT

5 Effects of plant growth stage and leaf aging on the response of transpiration and
6 photosynthesis to water deficit in sunflower

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24

25 **Abstract**

26 Water deficit influences leaf transpiration rate and photosynthetic activity. The
27 genotype-dependent response of the latter has not been assessed in sunflower
28 (*Helianthus annuus* L.), particularly during the reproductive period when grain
29 filling and lipogenesis depend greatly on photosynthate availability. To evaluate
30 genotypic responses to water deficit before and after flowering, two greenhouse
31 experiments were performed. Four genotypes – two inbred lines (PSC8, XRQ) and
32 two cultivars (Inedi, Melody) – were subjected to progressive water deficit. Non-
33 linear regression was used to calculate the soil water deficit threshold (FTSWt) at
34 which processes (transpiration and photosynthetic activity) were affected by water
35 deficit. In the vegetative growth stage, photosynthetic activity was affected at a lower
36 mean value of FTSWt (0.39) than transpiration (0.55). However, in the reproductive
37 stage, photosynthetic activity was more sensitive to soil water deficit (FTSWt =
38 0.45). We found a significant ($p = 0.02$) effect of plant growth stage on the difference
39 between photosynthesis and transpiration rate thresholds and, a significant ($p = 0.03$)
40 effect of leaf age on transpiration. Such results will improve phenotyping methods
41 and provide paths for integrating genotypic variability into crop models.

42 Keywords: genotype, net CO₂ assimilation rate, senescence, transpiration, water
43 stress, *Helianthus annuus* L.

44

45 **1. Introduction**

46 Water deficit is a major factor limiting the cultivation of sunflower (*Helianthus*
47 *annuus* L.) in southern Europe. Sunflower is cultivated during the summer, when
48 evaporative demand is high, particularly during grain filling. In addition, sunflower
49 is confined mostly to shallow soils, where water scarcity often occurs (Casadebaig,
50 2008; Terres Inovia, 2015). Even though sunflower is deemed tolerant to water
51 deficits, it must be managed properly to optimize grain production and quality
52 (Champolivier et al., 2011; Andrianasolo et al., 2016a; Andrianasolo et al., 2016b).

53 Patterns of water deficit response in sunflower are similar to those of most cultivated
54 species (Hsiao, 1973; Chaves et al., 2002). They consist of early and progressive leaf
55 stomatal closure due to high evaporative demand in the atmosphere and/or soil
56 dryness and loss of leaf turgor. Stomata are likely regulated by abscisic acid and
57 hydraulic signaling (Chaves et al., 2002; Pantin et al., 2012). At a moderate water
58 deficit, photosynthetic activity decreases mainly due to stomatal closure. The decline
59 in intercellular CO₂ following stomatal closure may induce a down-regulation of
60 biochemical demand for carbon dioxide (Chaves et al., 2002). Connor and Hall
61 (1997) reported that mechanisms involved in sunflower response to water stress
62 varied with growth stage, suggesting that the control of plant water status through
63 stomatal conductance changed with plant ontogeny (Pantin et al., 2012) before
64 interacting with senescence processes. Since grain development and oil accumulation
65 depend on available carbohydrates that originate mainly from photosynthetic activity
66 after flowering (Merrien, 1992), any impairment of photosynthesis due to water
67 deficit could likely reduce grain production and oil content. The sensitivity of
68 photosynthesis to water deficit and its relationship to stomatal or non-stomatal
69 limitations after flowering still needs to be investigated in sunflower.

70 Previous experiments have helped to highlight genetic variability in sunflower for
71 photosynthetic processes and plant water status in response to water deficit (Maury et
72 al., 1996; Maury et al., 2000; Kiani et al., 2007a; Kiani et al., 2007b). Genotypic
73 differences were also found for transpiration response to water deficit (Casadebaig et
74 al., 2008) in experiments in which plant water status was expressed as a fraction of
75 transpirable soil water (FTSW). This method was established by Sinclair and Ludlow
76 (1986), and later became routine for evaluating genotype and/or species response to
77 progressive water deprivation (Liu et al., 2005; Pellegrino et al., 2006; Casadebaig et
78 al., 2008; Verhoef and Egea, 2014). From the comparison of 25 different breeding
79 generation sunflower genotypes, Casadebaig et al. (2008) distinguished two
80 categories of responses to water deficit. The first, called the “conservative” strategy,
81 consisted of an “early” stomatal closure at moderate water deficit (*i.e.* when the
82 fraction of transpirable soil water is still high), leading to water conservation at the
83 expense of photosynthesis. A second strategy, referred to as “productive”, was
84 characterized by a “later” stomatal closure, thereby allowing it to maintain prolonged
85 photosynthetic activity (other things being equal). The delay between the start of
86 stomatal closure (transpiration response) and its influence on photosynthesis is
87 decisive for the potential productivity of a given genotype. In the SUNFLO crop
88 model, Casadebaig et al. (2011) assumed that photosynthetic activity was influenced
89 by water deficit after transpiration rate, irrespective of the plant growth stage and the
90 genotype; they used an offset parameter value to distinguish the processes. We
91 investigated whether this delay between transpiration and photosynthetic activity
92 varies with genotype and/or plant growth stage.

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

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

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

116 **2. Materials and methods**

117 2.1. Experimental design

118 Two greenhouse experiments were performed at the Institut National de la Recherche
119 Agronomique (INRA) station in Auzeville, France (43°31'41.8" N, 1°29'58.6" E) in
120 2009 (Exp.I) and 2012 (Exp.II). In Exp.I, two inbred lines were used (XRQ and
121 PSC8); their contrasting behaviors under water deficit had been previously
122 determined (Rengel et al., 2012). In Exp.II, XRQ and two commercial F1 hybrids
123 (Inedi and Melody) were used. Only XRQ was present in both experiments. Seeds
124 were germinated in Petri dishes, and plantlets were rapidly transferred to large 15 L
125 individual pots filled with a mixture of 50% clay loam, 40% P.A.M.2 potting soil
126 (Proveen, distributed by Soprimex, Chateaurnaud, Bouches-du-Rhône, France) and
127 10% sand. Seeds were sown on 1 April in 2009; two sowing dates were set in the
128 2012 experiment to obtain similar environmental conditions to simultaneously
129 monitor vegetative and reproductive growth stages: 24 April and 16 March,
130 respectively. Pots were randomly distributed within the greenhouse, and replicates
131 were grouped into several blocks. There were 5 (2009) and 6 blocks/replicates (2012)
132 and a total of 20 and 72 pots in 2009 and 2012, respectively. Plants were adequately
133 irrigated and fertilized (Rengel et al., 2012; Marchand et al., 2013) in both
134 experiments before water deficit was begun. Relative humidity and temperature of
135 the air inside the greenhouse were recorded using thermo-hygrometers (ROTRONIC
136 MP100A Temperature and Relative Humidity Probe, Campbell Scientific Ltd.,
137 Campbell Park, UK). Evaporative demand was estimated by calculating the vapor
138 pressure deficit (VPD) according to Tetens (1930). Global radiation above the
139 greenhouse was also monitored (CE-180, Cimel, France). Mean hourly
140 photosynthetically active radiation was 72.4 and 114.0 J cm⁻² in Exp.I and Exp.II,
141 respectively.

142 2.1.1. Water deficit treatment

143 After reaching the 8-leaf stage (as for Casadebaig et al. (2008) and Marchand et al.
144 (2013)) in the Exp.I and Exp.II “vegetative growth stage” experiment and the full
145 flowering stage (R5.5, Schneiter and Miller, 1981) in the Exp.II “reproductive
146 growth stage” experiment, we paired pots into non-irrigated/irrigated treatments. All
147 pots were irrigated to full soil water saturation capacity the day before the water
148 deficit experiment; no more water was provided to non-irrigated plants until the end
149 of the experiment. Irrigated pots were re-watered daily to full soil water saturation
150 capacity (200-700 ml, depending on daily evaporative demand and water
151 consumption). All pots were covered with a 3 mm layer of polystyrene sheets to
152 prevent soil evaporation. Soil evaporation was accounted for and estimated according
153 to Marchand et al. (2013).

154 2.1.2. Measurements

155 2.1.2.1. Leaf transpiration

156 Transpiration rate at single-leaf level (TL, $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) was measured within
157 2-3 min after closing the clamp of a porometer (LI-1600, LI-COR Inc., Lincoln, NE,
158 USA) in Exp.I and a portable gas-exchange system (LI-COR 6400, Lincoln,
159 Nebraska, NE, USA) in Exp.II. VPD was 1.9 ± 0.2 (mean \pm standard deviation) in
160 Exp.I and 1.2 ± 0.1 kPa in Exp.II. Temperatures were $26.8 \pm 1.1^\circ\text{C}$ in Exp.I and 25.5
161 $\pm 0.4^\circ\text{C}$ in Exp.II. PPFD was higher than $800 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (natural sunlight) in Exp.I
162 and $\sim 1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (LI-COR 6400-02 light source, Lincoln, Nebraska, NE,
163 USA) in Exp.II.

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

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

187 Table.1

188 2.1.2.2. Leaf net photosynthesis

189 Leaf net photosynthesis rate (PA, $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) of the reference “mature” leaf
190 was measured with the LI-COR 6400 device (Exp.II). Measurements were performed

191 under a saturated PPFD ($\sim 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$). Mean temperature and relative
192 humidity during photosynthetic activity measurements were $25.5 \pm 0.4^\circ\text{C}$ and $40 \pm$
193 4% , respectively. Carbon dioxide concentration was $400 \mu\text{mol mol}^{-1}$. As done for
194 leaf transpiration, leaf net photosynthesis was converted into a normalized ratio
195 between net photosynthetic activity of non-irrigated and irrigated plants (Normalized
196 Photosynthetic Activity rate at single-leaf level, NPA).

197 2.1.2.3. Daily plant transpiration

198 Plant transpiration was estimated from daily measurements of pot weight and leaf
199 area, following the method of Casadebaig et al. (2008). Daily weighings occurred at
200 5 p.m. and lasted 30 minutes. When a leaf displayed more than 50% senescence
201 (yellowing or browning), its area was discarded from the daily leaf area
202 measurement. Whole-plant transpiration rate (TP, $\text{kg m}^{-2} \text{day}^{-1}$) was obtained by
203 dividing daily water loss by plant leaf area. Plant transpiration values were
204 normalized (Normalized Transpiration rate at whole-Plant level, NTP) as the ratio
205 between non-irrigated and irrigated values for further comparison of water use
206 dynamics.

207 2.1.2.4. Water deficit experienced by the plant

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

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

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

216 2.2. Modeling the response of transpiration and photosynthesis to soil water deficit
217 and statistical analysis

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

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

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

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

239 **3. Results**

240 3.1. Comparison of single-leaf and whole-plant transpiration rates and photosynthetic 241 activity

242 Transpiration rates of mature leaves of XRQ at vegetative growth stage (TL, Table.2)
243 were higher for irrigated plants in Exp.I than in Exp.II (12.90 vs. 5.94 mmol m⁻² s⁻¹,
244 respectively). Transpiration rates at whole-plant level (TP) were also higher in Exp.I
245 than in Exp.II. Photosynthetic activity rates (PA) ranged from 1.28 to 15.85 μmol
246 CO₂ m⁻² s⁻¹ depending on the water status and growth stage. For the three processes
247 (PA, TL, TP), values for non-irrigated plants were always significantly lower than
248 those for irrigated plants.

249 Table.2

250 3.2. Responses of single-leaf transpiration and photosynthesis to soil water deficit in 251 vegetative and reproductive stages

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

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

270 Fig.1

271 Table.3

272 Table.4

273 Table.5

274 3.3. Response of transpiration rate at the single-leaf level to soil water deficit
275 depends on leaf age

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

280 response to soil water deficit of “mature” leaves was less sensitive (mean FTSW_t =
281 0.24 and 0.46 for PSC8 and XRQ, respectively; Table.7), and the lower sensitivity of
282 PSC8 contributed to significant genotypic differences in *a*.NTL.

283 Fig.2

284 Table.6

285 Table.7

286 3.4. Correlations between transpiration rates at single-leaf and whole-plant levels
287 When investigating the relationship between NTL and NTP in Exp.I, NTL was
288 significantly correlated with NTP for all leaf ages (Fig.3). “Post-maturing” and
289 “mature” leaves had the strongest relationships ($R^2 = 0.67$ and 0.68 , respectively). R^2
290 was on average lower in Exp.I (0.65) than in Exp.II (0.83); in the latter, NTP and
291 NTL had a slightly stronger relationship in the vegetative growth stage (Fig.4).

292 Fig.3

293 Fig.4

294 **4. Discussion**

295 *Methodological limitations and potential interactions with the growth environment*

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

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

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

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

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

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

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

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

366 *Existing genotypic differences requiring further investigation*

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

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

388 **5. Conclusions**

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

399

400 **6. Acknowledgments**

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406

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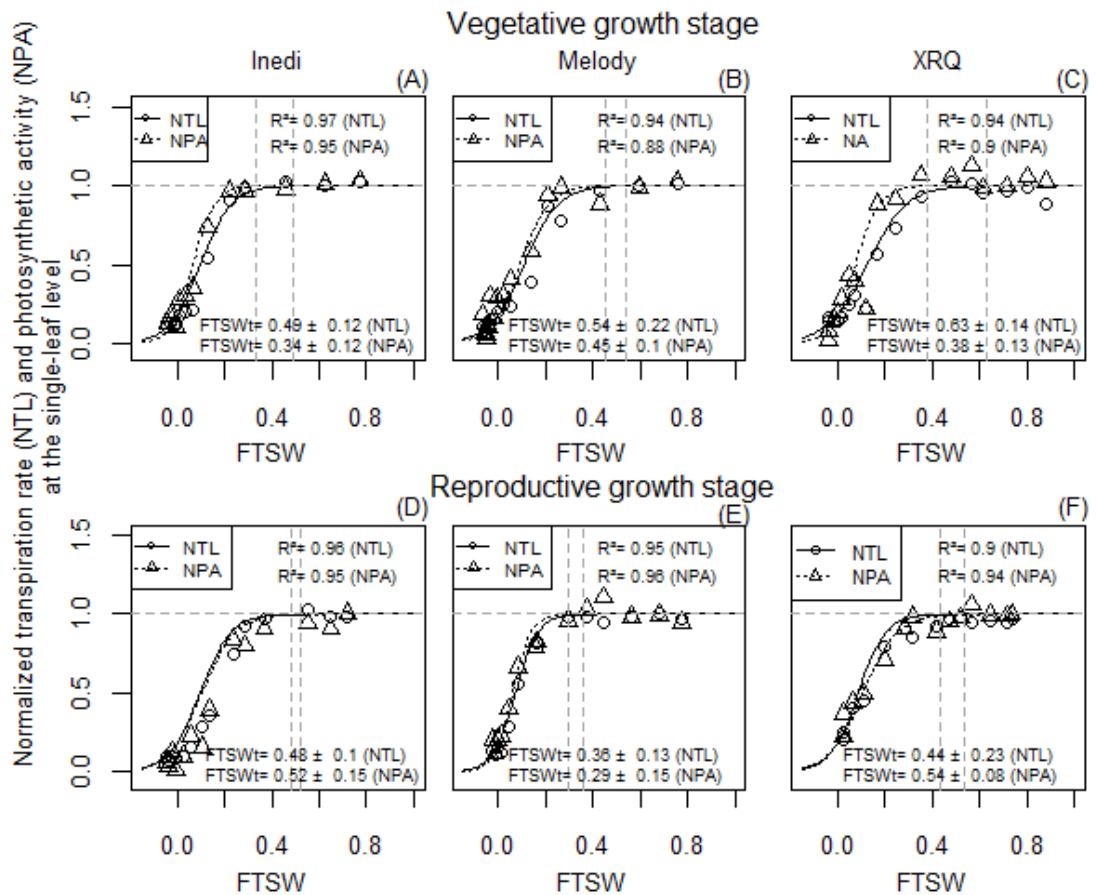
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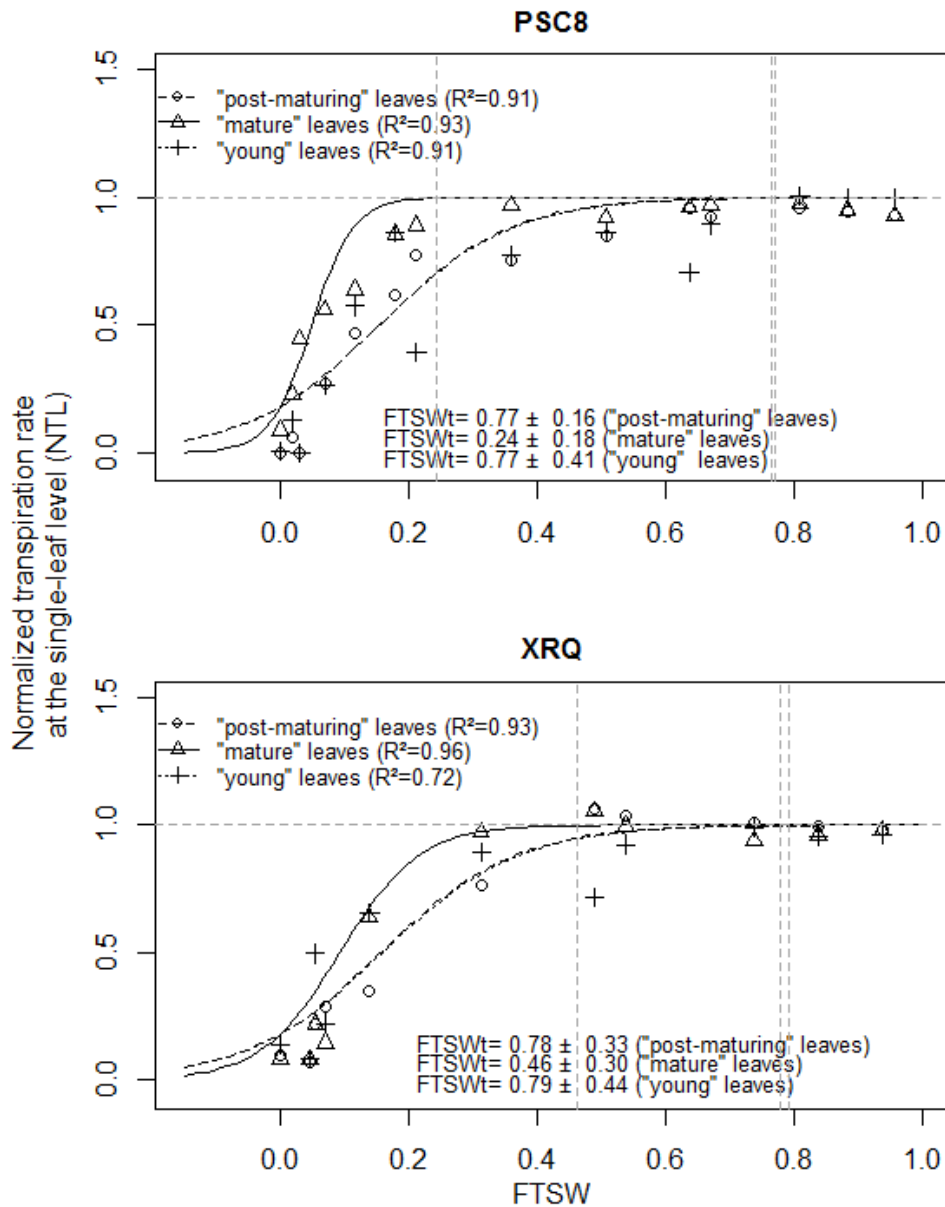
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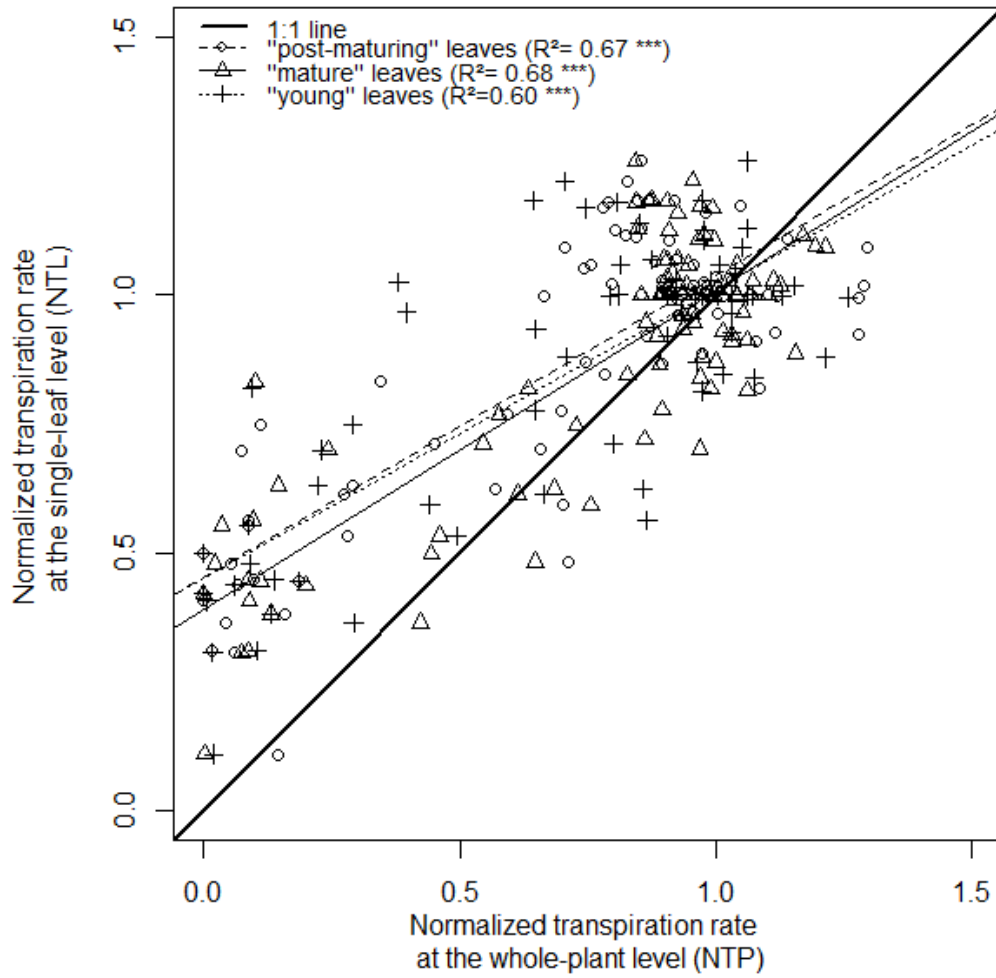
506 Fig.1. Responses of normalized transpiration rate (NTL) and photosynthetic activity
 507 (NPA) at the single-leaf level to fraction of transpirable soil water (FTSW) in (A, B,
 508 C) vegetative and (D, E, F) reproductive growth stages for 3 genotypes (Inedi,
 509 Melody, XRQ) during Exp.II. Quality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

512



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 \pm 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.

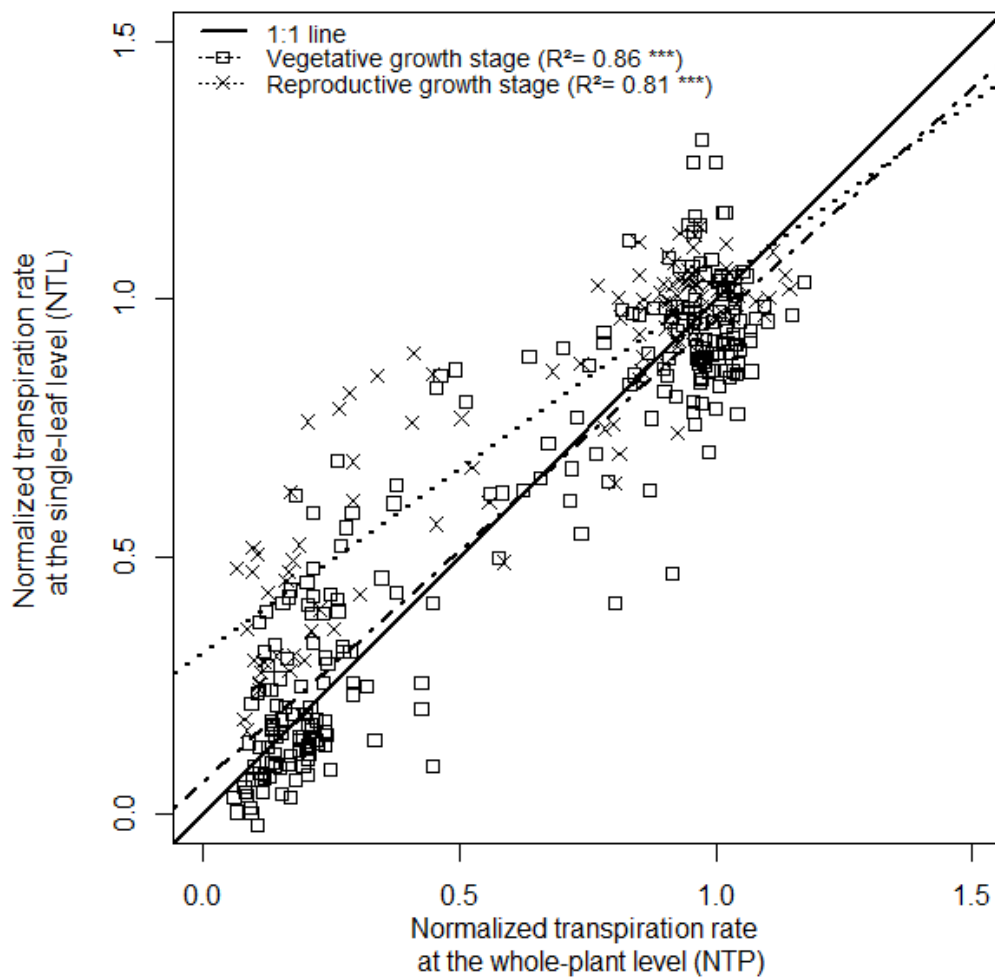


522

523 Fig.3. Relationship between normalized transpiration rates at single-leaf (NTL) and

524 whole-plant (NTP) levels in Exp.I. Relationships were assessed by leaf age.

525



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 ($^{\circ}\text{C}$ -days from initiation) used (\bullet)
 532 in Exp.I and Exp.II. n = not used

533

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

534

535 Table.2. Means \pm 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

542

Plant growth stage	Leaf name	PA ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)		TL ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)		TP ($\text{kg m}^{-2} \text{ day}^{-1}$)	
		Non-irrigated	Irrigated	Non-irrigated	Irrigated	Non-irrigated	Irrigated
Exp.I							
Vegetative	"Post-maturing"	n/a		2.40 \pm 1.51 a	11.23 \pm 2.20 b	4.40 \pm 1.70 a	5.90 \pm 0.40 b
	"Mature"			2.97 \pm 2.78 a	12.90 \pm 2.30 b		
	"Young"			3.80 \pm 2.33 a	8.26 \pm 1.62 a		
Exp.II							
Vegetative	"Mature"	1.28 \pm 0.81 a	15.85 \pm 2.28 b	0.81 \pm 0.04 a	5.94 \pm 0.80 b	0.13 \pm 0.09 a	1.05 \pm 0.03 b
Reproductive		4.25 \pm 3.17 a	13.44 \pm 2.74 b	1.99 \pm 0.88 a	5.97 \pm 0.75 b	0.58 \pm 0.22 a	1.48 \pm 0.17 b

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 R²) 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	<i>a</i>	-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	<i>a</i>	-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	<i>a</i>	-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	<i>a</i>	-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	<i>a</i>	-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	<i>a</i>	-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.

<i>a</i> .NTL- <i>a</i> .NPA	df	Sum Sq	Mean Sq	F	p
Source of variation					
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 \pm 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.

<i>a</i> .NTL- <i>a</i> .NPA	Growth stage	
Genotype	Vegetative	Reproductive
Inedi	7.00 \pm 7.91 ab	-2.17 \pm 7.20 bc
Melody	2.55 \pm 2.50 abc	4.52 \pm 5.99 ab
XRQ	7.85 \pm 5.62 a	-3.14 \pm 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 $p < 0.05$.

<i>a</i> .NTL	df	Sum Sq	Mean Sq	F	p
Source of variation					
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

569 Table.7. Summary table of response parameters to water deficit for normalized
 570 transpiration rate at single-leaf and whole-plant levels (NTL and NTP respectively)
 571 during Exp.I. Fitted values of a , fraction of transpirable soil water (FTSWt) values
 572 and indicators of quality of fit (root mean squared error (RMSE) and R^2) are
 573 presented per genotype and leaf age. The a parameter describes the shape of the
 574 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

575