

1 **Phenolic profile and content of sorghum grains under different irrigation managements**

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17 **Abstract:**

18 Sorghum grain is widely consumed in Sub-Saharan Africa and Asia, as a staple food
19 due to its adaptation to harsh environments. The impact of irrigation regime: full
20 irrigation (100%); deficit irrigation (50%); and severe deficit irrigation (25%) on
21 phenolic profile and content of six sorghum grain genotypes was investigated by high
22 performance liquid chromatography coupled with diode array detection and
23 electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS). A total of 25
24 individual polyphenols were unequivocally or tentatively identified. Compared to the
25 colored-grain genotypes, the white grained sorghum var. Liberty had a simpler
26 polyphenol profile. The concentrations of the sorghum-specific
27 3-deoxyanthocyanidins luteolinidin and apigeninidin, were higher under deficit
28 irrigation compared to the other two regimes in all genotypes. These findings will be
29 valuable for the selection of sorghum genotypes for grain production as human food
30 under water deficit conditions, since polyphenol levels can affect the grain's
31 nutritional value and health properties.

32 **Keywords:** *sorghum; genotype; irrigation; polyphenols; HPLC-MS*

33 **1. Introduction**

34 Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most valuable global cereal crop,
35 widely grown in semi-arid and arid regions of the world because of its tolerance to
36 drought and high temperatures (Taylor, Schober, & Bean, 2006). In many parts of
37 Africa and Asia, sorghum grain provides nutrients and energy for millions of local
38 people, whereas in the developed countries such as the USA and Australia, it is used
39 primarily as an animal feed or for biofuel production (Stefoska-Needham, Beck,
40 Johnson, & Tapsell, 2015). However, the number of people consuming sorghum grain
41 is slowly but steadily increasing in developed countries mainly due to sorghum's
42 gluten-free property and antioxidant potential from polyphenolic phytochemicals
43 (Taylor et al., 2006).

44 Polyphenols have antioxidant activity due to their free-radical scavenging
45 capability, and thus may protect against some chronic diseases, such as coronary heart
46 disease and type 2 diabetes (Dykes & Rooney, 2007). Polyphenols in sorghum grain
47 consist of simple phenolic acids (e.g. ferulic and *p*-coumaric acids),
48 3-deoxyanthocyanidins, flavanones, flavones and other flavonoids, as well as
49 condensed tannins (Awika & Rooney, 2004). In particular, the 3-deoxyanthocyanidins,
50 including apigeninidins, luteolinidins, 5-methoxyluteolinidin and
51 7-methoxyapigeninidin, are at high levels in some sorghum grain genotypes, but are
52 absent in other cereal grains (Awika & Rooney, 2004; L Dykes & Rooney, 2007). The
53 amounts and profiles of polyphenols in sorghum grain vary significantly between
54 genotypes. For example, it has been reported that red and yellow sorghum genotypes

55 contained high amounts of flavones, and sorghum genotypes with pigmented testa
56 have higher content of condensed tannins (Taleon, Dykes, Rooney, & Rooney, 2014;
57 Wu et al., 2016a).

58 Under a changing climate, annual mean precipitation is projected to decrease in
59 many mid-latitude and subtropical dry regions, in which crops, such as sorghum,
60 maize and pearl millet, will invariably suffer from moisture stress (Pachauri et al.,
61 2014). Polyphenol content and antioxidant activity of plant materials may be affected
62 by water deficit, and their changes depend on plant species (Cohen & Kennedy, 2010).
63 Tovar, Motilva, and Romero (2001) planted young olive trees under seven irrigation
64 treatments. They found that the concentration of the dialdehydic form of elenolic acid
65 and oleuropein aglycon of the olive oils and the antioxidant activity significantly
66 increased as the amount of irrigation water decreased to deficit levels. Buendía,
67 Allende, Nicolás, Alarcón, and Gil (2008) investigated the effects of regulated deficit
68 irrigation and full irrigation on polyphenols and antioxidant activity of peaches, and
69 reported that the content of phenolics, mainly anthocyanins and procyanidins, and
70 antioxidants increased under regulated deficit irrigation. In another study, comparing
71 irrigated and non-irrigated grapevines, the levels of proanthocyanidins and flavonols
72 increased in fruit from irrigated vines (Zarrouk et al., 2012). There is little information
73 in the literature from controlled studies investigating how level of irrigation
74 influences profile and concentrations of polyphenols of sorghum grain. In our recent
75 study, it was found that the levels of total polyphenol and antioxidant activity of
76 sorghum grain significantly increased when the amount of water was reduced (Wu,

77 Johnson, Bornman, Bennett, & Fang, 2017). However, individual polyphenols of
78 sorghum grain were not measured in the previous study, and it is also still unknown
79 how irrigation treatment influences the profile of polyphenols in sorghum grain.

80 Therefore, in the present study, using an as yet unreported trial, the effects of three
81 levels of irrigation treatments on the individual phenolic compounds of six different
82 sorghum genotypes were determined by the powerful analytical technique of high
83 performance liquid chromatography coupled with diode array detection and
84 electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS).

85 **2. Materials and methods**

86 *2.1. Plant material and treatments*

87 The sorghum field experiment was conducted at Curtin University's Field Trials
88 Area, Western Australia (latitude 32°00'S, longitude 115°53'E, altitude 20 m). Daily
89 rainfall and minimum/ maximum air temperature were obtained from the Perth
90 Airport Bureau of Meteorology weather station 9.6Km away from the experimental
91 site (Supplementary Fig S1) (BOM, 2013).

92 Six sorghum genotypes comprised of two hybrid lines ('Liberty' white pericarp and
93 'MR Bazley' red pericarp) and four inbred lines ('Alpha' red pericarp; 'IS1311C' and
94 'IS8237C' both brown pericarp; and 'Shawaya Short Black 1', dark red-black
95 pericarp). All seeds were provided from the Australian sorghum pre-breeding program,
96 a partnership between the University of Queensland, the Queensland Department of
97 Agriculture and Fisheries and the Grains Research and Development Corporation,
98 courtesy of Professor David Jordan. All samples were planted in 1 m x 1 m fibre glass

99 pots with a depth of 0.5 m. One row each of three sorghum genotypes were planted in
100 each pot, with a row spacing of 0.25 m. Each row was sown on 9th January 2014 with
101 10 seeds of the nominated variety and thinned to five plants spaced 0.2 m apart after
102 two weeks. The experiment of 6 genotypes x three levels of irrigation was carried out
103 in two replications with a randomised complete block design.

104 The potential reference crop evapotranspiration (PET_0) from the nearby weather
105 station was 822.7 mm from sorghum sowing date to maturity 10th May. In the same
106 period, the crop potential evapotranspiration under standard conditions (PET_c) was
107 calculated from PET_0 and the Food and Agricultural Organization (FAO) crop
108 coefficient (K_c) for sorghum, giving a PET_c of 576.25mm (Allen, Pereira, Raes, &
109 Smith, 1998). The experimental irrigation implementation was based on PET_c . Three
110 irrigation regimes were applied: full irrigation (FI, 100% PET_c), deficit irrigation (DI,
111 50% PET_c) and severe deficit irrigation (SDI, 25% PET_c).

112 The sowing date was defined as 0 day after sowing, and all plants received
113 unlimited water in the first two weeks. After that, all irrigation treatments were
114 applied by hand watering. Sorghum was irrigated every 3–4 days with a total of 24
115 irrigations during the growing season. All grains were harvested at maturity, air-dried
116 to a moisture content of around 10%, manually cleaned, vacuum packed and stored at
117 -20°C until analysis.

118 2.2. *Physical characteristics of grain*

119 The Single Kernel Characterization System (SKCS 4100, Perten Instruments,
120 Hågersten, Sweden) was used to evaluate the physical characteristics of sorghum

121 grain, and all samples were evaluated in duplicate.

122 *2.3. Phenolic extraction*

123 All sorghum whole grains were milled to pass 100% through a 500 µm sieve using a
124 grain mill (CEMOTEC 1090, Foss Tecator, Hoganäs, Sweden). For free and bound
125 polyphenols, extraction was conducted according to the method of Svensson,
126 Sekwati-Monang, Lutz, Schieber, & Gänzle (2010) with some modifications. In brief,
127 the 15 mL of 80% (v/v) aqueous methanol was mixed with around 2 g of the ground
128 sample under N₂, and the mixture was shaken in the water bath at 25°C for 2 h. The
129 supernatant was collected after centrifuging at 3,220 × g for 10 min at 4°C. The
130 residue was extracted with 20 mL 80% (v/v) aqueous methanol two times more, and
131 all supernatants were combined after centrifuging. Rotary vacuum evaporation was
132 carried out to evaporate supernatants to dryness. The resulting solid was re-dissolved
133 in 10 mL of methanol and stored at -20°C under N₂. The residue remaining after free
134 polyphenol extraction was the used for bound phenolic extraction.

135 For the extraction of bound phenolic compounds, the residue after free polyphenol
136 extraction was mixed with 15 mL of 2 M hydrochloric acid (HCl) under N₂ in the
137 water bath at 100°C for 1 h. After hydrolyzing, the 15 mL ethyl acetate was added and
138 thorough mixed. Then, the ethyl acetate fraction was collected after partitioning. The
139 hydrolysate was re-extracted with the 15 mL ethyl acetate four times more, and all
140 ethyl acetate fractions were combined and evaporated to dryness. The resulting solid
141 was re-dissolved in 10 mL of methanol and stored at -20°C under N₂ before
142 analysing.

143 *2.4. HPLC-DAD-ESI-MSⁿ analysis*

144 An Agilent 1290 UHPLC system with diode array detector (DAD) and Agilent
145 6460 LC-MS/MS System (Agilent Technologies, Palo Alto, CA, USA) were
146 used to separate polyphenols according to the procedure described in detail previously
147 (Wu et al., 2016b). Briefly, the Kinetex XB-C 18 reversed phase-HPLC column (5 μ m,
148 250 \times 4.6 mm, Phenomenex, Torrance, CA, USA) was used to separate individual
149 phenolic compounds, and the scanning range of the DAD was set between 190 and
150 600 nm at steps of 2 nm. Solvent A was 0.1% formic acid in LC-MS grade water
151 (Honeywell Burdick & Jackson, Gillman, SA, Australia), and solvent B consisted of
152 LC-MS grade acetonitrile (Honeywell Burdick & Jackson, Gillman, SA, Australia).
153 Extract (5 μ L) was injected and the following linear gradient elution was used: 5%-15%
154 B (5 min), 15%-50% B (40 min), 50%-70% B (2 min), 70%-100% B (1 min), 100% B
155 (7 min), 100%-5% B (1 min), 5% B (9 min). The rate of flow was 0.5 ml/min.
156 Mass spectra were performed in the ESI negative mode with a scan time of 2000 MS
157 under the following conditions: gas (N₂) 5 L/min at 300 °C, nebulizer 45 psi, sheath
158 gas (N₂) 11 L/min at 250°C, capillary voltage -3.5kV and nozzle voltage -500V.
159 Phenolic compounds were detected by full scan ranging from m/z 50 to 1300.

160 *2.5. Quantification of polyphenols*

161 The following individual authentic standards were used for quantitation. Ferulic
162 acid, caffeic acid, luteolin, apigenin, taxifolin and naringenin were purchased from
163 Sigma–Aldrich (St. Louis, MO, USA). Luteolinidin chloride, and apigeninidin
164 chloride were purchased from Alsachim (Strasbourg, France). Results were expressed

165 as $\mu\text{g/g}$ sample (db). All extracts were analysed in duplicate.

166 2.6. Statistical analysis

167 All data were reported as means \pm standard deviation (SD) using SPSS Statistics
168 V20 (IBM Corp., Armonk, NY, USA). The main effects of genotype and irrigation
169 and their interaction were analysed using a two-way ANOVA with Tukey post-hoc test.
170 $P \leq 0.05$ was considered significantly different. A principal components analysis was
171 run on the complete dataset for each irrigation treatment using Statistica 10 (Dell
172 Software., Tulsa, Oklahoma, USA). A correlation matrix across all the variables
173 within an irrigation treatment was run to check if any of the variables were highly
174 correlated with any of the other variables prior to running the PCA. A plot of principal
175 components 1 and 2 (PC1 and PC2) for each irrigation treatment enabled the
176 genotypes to be clustered into a number of groups based on their expression of the
177 polyphenolic compounds. An analysis of variance was used to test the significance of
178 these groups.

179 3. Results

180 3.1. Kernel physical characteristics

181 Genotype, irrigation regime and their interaction had a significant influence on
182 weight and diameter of sorghum grain (Fig. 1) ($P \leq 0.05$). The values of grain weight
183 and diameter were significantly reduced when reducing the amount of irrigation in all
184 sorghum genotypes. Sorghum Shawaya Short Black 1 had the highest values of grain
185 weight and diameter under the three irrigation regimes, while the lowest values were
186 in sorghum IS8237C under FI and DI regimes and Alpha under SDI regime.

187 *3.2. Identification of polyphenols*

188 Individual phenolic compounds in the six sorghum genotypes from three irrigation
189 regimes were monitored by HPLC-DAD and mass spectrometry (MS) detection.
190 Representative HPLC chromatograms of phenolic compounds in the genotype of MR
191 Bazley are presented in Fig 1. The MS and UV characteristics of the chromatographic
192 peaks from all samples, along with their proposed structure, are shown in Table 1. The
193 25 different phenolic compounds were identified in the extracts. A total of eight
194 individual polyphenols, including ferulic acid, caffeic acid, luteolin, apigenin,
195 luteolinidin, apigeninidin, taxifolin and naringenin, were unequivocally identified and
196 another 17 tentatively identified.

197 Peaks, **8, 9, 13, 16, 17, 21, 24** and **25** were identified by authentic standards based
198 on their chromatographic comparisons. Peaks **2, 5-7, 11, 14-15, 18-20** and **22-23** were
199 identified in detail in our previous study (Wu et al., 2016b). Of the other peaks, Peak
200 **1** represents an unknown complex polyphenol. It was noted that peak **3** had similar
201 UV and MS spectra to peaks **18** and **19**, and was tentatively identified as
202 1,2-*O*-dicaffeoylglycerol or 1,3-*O*-dicaffeoylglycerol isomer. Peak **4** had a
203 deprotonated molecule $[M - H]^-$ ions at m/z 353, and prominent fragment ions at m/z
204 191 and 173, corresponding to deprotonated quinic acid and caffeic acid fragments,
205 respectively. After comparisons with the literature (Han et al., 2008), peak **4** was
206 tentatively identified as chlorogenic acid (5-caffeoylquinic acid). Peak 10 had a
207 deprotonated molecule $[M-H]^-$ at m/z 447. The $[M - H - 162]^-$ ion at m/z 285 is typical
208 of that produced by the loss of a hexoside residue, and the prominent fragment ions at

209 m/z 285 would correspond to the deprotonated luteolin fragment. Hence, peak **10** was
210 tentatively identified as luteolin hexoside. Peak **12**, with a deprotonated molecule
211 $[M-H]^-$ at m/z 433, also lost a hexoside residue (433- 162) to produce the prominent
212 fragment ions at m/z 271, which could correspond to deprotonated naringenin
213 fragment. Therefore, peak **12** was tentatively identified as naringenin hexoside (Table
214 1).

215 *3.3. Influence of genotype and irrigation regime on the profiles of polyphenols of* 216 *grains*

217 The representative HPLC chromatograms of free polyphenols in the MR-Bazley
218 genotype are shown in Fig 1. The HPLC chromatograms illustrated that across all
219 genotypes, irrigation treatments did not differ in the polyphenolic species present
220 rather only altered their concentrations. The profiles of both free and bound forms of
221 individual polyphenols of six sorghum genotypes under three irrigation regimes are
222 shown in Supplementary Table S2.

223 *3.4. Influence of genotype and irrigation on the individual polyphenol concentration* 224 *of grains*

225 It is important to understand the presence and concentrations of individual
226 polyphenols for specific applications of the grain, such as for high antioxidant health
227 foods (Yousif, Nhepera, & Johnson, 2012), as the antioxidant properties of sorghum
228 grain relate to both polyphenol concentration and profile (Wu et al., 2016b; Wu et al.,
229 2016c). In the present study eight phenolic compounds were quantified based on the
230 availability of authentic standards. Based on their structural characteristics, the

231 quantified phenolics were classified into five groups and their concentrations
232 determined.

233 *Hydroxycinnamic acids.* The hydroxycinnamic acids quantified were caffeic acid
234 and ferulic acid (Table 2). The free, bound and total levels of these compounds were
235 significantly affected by genotype, irrigation regime and their interaction ($p \leq 0.05$).
236 Under the FI regime, the highest and lowest free and total caffeic acid concentrations
237 were found in Alpha and IS1311C, respectively. For total ferulic acid concentration
238 under the FI regime, IS8237C had the lowest while MR-Bazley had the highest. Water
239 deficit treatments significantly increased the level of free ferulic acid as compared to
240 the FI treatment. A reduction in the amount of irrigation water resulted in varying
241 changes in both bound caffeic acid and ferulic acid concentration amongst the
242 genotypes. Under the SDI regime, Liberty had the lowest concentrations of total
243 caffeic acid and ferulic acid, while IS1311C and MR-Bazley had the highest total
244 caffeic acid and total ferulic acid contents, respectively.

245 *3-Deoxyanthocyanidins.* The concentration of luteolinidin and apigeninidin is
246 presented in Table 3. Again, the concentrations of these compounds were significantly
247 influenced by genotype, irrigation regime and their interaction ($p \leq 0.05$).
248 Luteolinidin and apigeninidin were not detected in the white sorghum, Liberty.
249 Otherwise the lowest detectable concentrations of free luteolinidin and apigeninidin
250 were in MR-Bazley and Alpha under FI regime, respectively, while Shawaya Short
251 Black 1 had the highest values of these two compounds. The intermediate DI regime
252 resulted in a higher concentration of luteolinidin than under the FI and SDI regimes.

253 The concentration of both bound luteolinidin and apigeninidin varied with decreasing
254 water supply and genotype Shawaya Short Black 1 had the highest contents of total
255 luteolinidin and apigeninidin under both FI and DI regimes, but became the lowest
256 under SDI regime when compared with other genotypes.

257 *Flavones.* Two flavones were quantified in the samples namely luteolin and
258 apigenin (Table 3), with significant changes in concentrations ($p \leq 0.05$) due to
259 genotype, irrigation regime and their interaction. No apigenin in free or bound form
260 was detected in Liberty or Alpha under the three irrigation regimes. Free and total
261 luteolin and apigenin concentrations were the highest ($p \leq 0.05$) under the DI regime
262 across all genotypes. Shawaya Short Black 1 contained the highest contents of free
263 and total luteolin, but the lowest contents of free, bound and total apigenin under FI
264 regime. However, differing irrigation regimes resulted in different levels of bound
265 luteolin and apigenin concentrations. Compared to FI and SDI regimes, the higher
266 contents of free, bound and total luteolin and apigenin were in all sorghum genotypes
267 under DI regime. Irrespective of treatment, the lowest contents of free, bound and
268 total apigenin were in Shawaya Short Black 1.

269 *Dihydroflavonol and Flavanones.* One dihydroflavonol taxifolin and one flavanone
270 naringenin were identified and quantified, and their concentrations were significantly
271 affected by genotype, irrigation regime and their interaction ($p \leq 0.05$), as shown in
272 Table 3. Free, bound and total taxifolin and naringenin concentrations were highest
273 under the intermediate DI regime when compared to the FI and SDI regimes across all
274 genotypes, with the exception of Liberty, where taxifolin and naringenin were not

275 detected. The lowest detected concentration of total taxifolin was in Alpha, while both
276 IS1311C and IS8237C had the lowest detected concentration of total naringenin under
277 the DI regime.

278 *3.5. Principal component analysis (PCA)*

279 A PCA of the six sorghum genotypes using the quantitative polyphenolic variables
280 found that 86.36%, 83.90% and 90.18% of the variation could be explained by the
281 first three components under FI, DI and SDI treatments, respectively, (Tables S4-S6).

282 A plot of PC1 and PC2 is shown in Figure 3, where it can be seen that a number of
283 individual polyphenols form distinct groups under three irrigation treatments. Three,
284 four and four groups were identified under FI, DI and SDI treatments, respectively.

285 Under FI treatment (Fig 3a), Group B (Shawaya Short Black 1) has a high level of
286 individual flavonoids, while Group A (Alpha and MR-Bazley) has high individual
287 hydroxycinnamic acids. For DI regime, a high level of individual flavonoids is

288 presented in Group B (Shawaya Short Black 1), but Group A (Alpha, MR-Bazley and
289 IS8237C) has low levels of individual polyphenols. Group C (Liberty) has a low level
290 of individual polyphenols, while a high level of some individual flavonoids is found

291 in Group B (Shawaya Short Black 1) or Group D (IS1311C). To evaluate the
292 significance of the groupings, an analysis of variance was run on both PC1 and PC2
293 with the groups listed above used as the treatment. Both PC1 and PC2 were shown to

294 have a significant difference between the groups ($p \leq 0.05$), across all of the irrigation
295 treatments.

296 **4. Discussion**

297 The processing quality of grain is principally contributed by grain physical
298 characteristics. The range of grain weight and diameter in the present study was in the
299 range of previous reports (Liu et al., 2013; Wu et al., 2008; Wu et al 2016c). However,
300 both Liu et al. (2013) and Wu et al. (2008) reported that the values of grain weight
301 and diameter were not significantly influenced by reduced irrigation levels. The
302 differences recorded in this study may be caused by the severe water stress on the SDI
303 treatment and the higher daytime temperatures present during the growing season
304 (Supplementary Fig S1).

305 Deficit irrigation had a significant influence on individual polyphenols. Some key
306 enzymes involved in the biosynthesis of individual polyphenols, have been previously
307 identified including phenylalanine ammonia lyase (PAL), chalcone synthase (CHS)
308 and flavonoid-3'-hydroxylase (F3'H) (Cohen & Kennedy, 2010). Synthesis of
309 3-deoxyanthocyanidin has been previously shown to be catalysed by CHS and F3'H
310 enzymes, and the synthesis of these two enzymes was reported to be enhanced under
311 biotic stress in sorghum, which led to increased 3-deoxyanthocyanidin concentration
312 (Boddu et al., 2004; Lo et al., 1999). In the present study, total 3-deoxyanthocyanidin
313 concentration was the highest under DI regime across all genotypes when compared
314 with other regimes. Therefore, it is proposed that more CHS and F3'H enzymes might
315 be synthesized when irrigation level was reduced from FI to DI. However, more
316 research work will be needed to confirm this hypothesis. Additionally, the effect of
317 water deficit on the metabolic pathways of other individual polyphenols is still
318 unknown in sorghum, so further research is required to understand these pathways.

319 In the present study, some individual polyphenols significantly decreased in
320 concentration when irrigation was decreased from DI to SDI regime. These findings
321 contrast with other reports, in which polyphenol concentration increased when the
322 level of irrigation decreased (Artajo, Romero, Tovar, & Motilva, 2006; Buendía et al.,
323 2008; Martinelli, Basile, Morelli, d'Andria, & Tonutti, 2012; Tovar et al., 2001).
324 Normal physiological and biochemical plant processes can be inhibited by severe
325 water deficit through reduced cell elongation and reduced photosynthesis (Jahanzad et
326 al., 2013; Saeed & El-Nadi, 1998). In the present study, sorghum grain yield was the
327 lowest under SDI treatment (data not shown), with no sorghum grain set in sorghum
328 IS8237C, which indicated that the SDI regime may have seriously inhibited
329 physiological and biochemical processes compared with FI and DI regimes. It is
330 proposed therefore that the synthesis of polyphenols might be also have been inhibited
331 under the SDI regime. Temperatures above 35°C have also been reported to decrease
332 the concentration of total and individual polyphenols in sorghum grain (Wu et al.,
333 2016b; Wu et al., 2016c). The average maximum daytime temperature during the
334 growth of the sorghum plants in the present study was in fact in excess of 35°C,
335 (Supplementary Fig S1). As the sorghum was unable to regulate the severe water
336 stress, the high temperature might also have decreased the biosynthesis of
337 polyphenols, flavonoids and some individual polyphenols on the sorghum grain under
338 the SDI regime. These findings might provide valuable information about polyphenol
339 concentrations in plants grown under a Mediterranean climate, such as the
340 Mediterranean Basin, and some regions of Australia, and North and South Africa, in

341 which plant biochemical and physiological processes are mainly regulated by water
342 deficit coupled with other interacting factors, such as high temperatures.

343 The profiles of individual polyphenols in the sorghum grain varied among the
344 different genotypes. Two different red sorghums: MR-Bazley and Alpha were planted
345 in this study, but only Alpha lacked apigenin (Supplementary Table S1). Taleon et al.
346 (2014) also supported that not all red sorghum genotypes contained luteolin.

347 The concentration of individual polyphenols determined in this study is in
348 agreement with previous studies (Dykes et al., 2009; Taleon et al., 2012; Wu et al.,
349 2016b). Black sorghum had the highest levels of 3-deoxyanthocyanidins when
350 compared to other genotypes, suggesting that black sorghum is a predominant source
351 of this group of compounds. Similar results have been reported by Taleon et al. (2012).
352 The accumulation of 3-deoxyanthocyanidins in sorghum grain is regulated by *PI*,
353 which is controlled by *Yellow seed1* gene (Ibraheem, Gaffoor, & Chopra, 2010). As no
354 3-deoxyanthocyanidins were detected in white sorghum Liberty, it is proposed that
355 *Yellow seed1* gene might be absent in this genotype. The luteolin concentration of the
356 two red genotypes, MR-Bazley and Alpha was significantly different from each other
357 (Table 3). Previously published data on two other red sorghum genotypes, Tx2911 and
358 98CA4779 reported that they contained 10.8 and 13.4 $\mu\text{g/g}$ luteolin, respectively
359 (Dykes et al., 2009), more than about twice higher than our values, which indicates
360 that the individual flavonoid concentration cannot be simply predicted by grain color.
361 These results highlight that before producing sorghum products with target levels of
362 specific polyphenols, such as 3-deoxyanthocyanidins for potential health benefits, it is

363 essential to evaluate the profile and concentration of the individual polyphenols in the
364 genotype being used.

365 PCA is one of the oldest and most widely used techniques for reducing the
366 dimensionality and increasing the interpretability of large datasets (Jolliffe & Cadima,
367 2016). The success of PCA can be seen in Rodríguez-Delgado, González-Hernández,
368 Conde-González, & Pérez-Trujillo (2012), who used PCA to understand the
369 polyphenol content of 55 samples of red wines from different locations, with all
370 samples separated into three groups according to geographical area of origin. In the
371 present study, classification of sorghum genotypes into several groups was possible
372 when a two-dimensional plot of the first two principal components was used to
373 evaluate the scores of each sorghum sample. It is also noticed that three of six
374 genotypes always fall in to the same groups: Group A and Group B. However, the
375 levels of polyphenols vary depending on the irrigation treatment (Fig. 3), suggesting
376 that irrigation treatment modifies polyphenol content of these genotypes. Dietary
377 intake of whole sorghum grain with abundant levels of polyphenols has potential to
378 reduce the risk of certain cancers, cardiovascular disease and type 2 diabetes, owing
379 to their antioxidant properties (Stefoska-Needham et al., 2015). The free, bound and
380 total antioxidant capacity in sorghum grains as affected by irrigation treatments was
381 evaluated by 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) and
382 the 2-2-diphenyl-1-picrylhydrazyl (DPPH) assays, and all data was presented in the
383 Supporting Information Table S3. In the present study, sorghum Shawaya Short Black
384 1 had the highest concentration of total polyphenols and flavonoids, followed by

385 IS8237C, a brown-coloured grain and these two genotypes also had higher
386 concentration of antioxidant activity (Supplementary Table S2 and S3). Recently,
387 several sorghum products, such as pasta (Khan, Yousif, Johnson, & Gamlath, 2013)
388 and extruded snack food (Licata et al., 2015), have been developed to enhance
389 antioxidant capacity of these food products. Khan, Yousif, Johnson, and Gamlath
390 (2015) reported that in comparison with 100% durum wheat pasta and pasta
391 containing 30% white wholegrain sorghum, the antioxidant status through increasing
392 plasma polyphenols, antioxidant capacity and superoxide dismutase activity was
393 increased post-prandially in healthy human participants after consuming pasta
394 containing 30% red wholegrain sorghum flour. It was suggested that these differences
395 may be due to the higher polyphenol content and the different profile of polyphenolic
396 compounds found in red wholegrain sorghum flour compared to white wholegrain
397 sorghum flour and durum wheat, probably due to the presence of
398 3-deoxyanthocyanidins in red wholegrain sorghum flour only (Khan et al., 2013).

399 **5. Conclusion**

400 In conclusion, irrigation, genotype and their interaction had a significant influence
401 on individual polyphenols, and most of them showed the highest concentration under
402 the intermediate DI regime. The present study suggested that both irrigation regime
403 and genotype should be considered when selecting sorghum genotypes to plant for the
404 production of high antioxidant value health foods and/or nutraceuticals.

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526

527 **Figure Captions**

528 Figure.1. Weight (a) and diameter (b) of sorghum grain as affected by irrigation
529 treatments.

530 ^{a-e} Values with different superscripts in the same irrigation treatment are significantly different
531 ($P \leq 0.05$). A, B Values with different superscripts in the same genotype with different
532 irrigation treatments are significantly different ($P \leq 0.05$).

533 Abbreviations: FI= full irrigation; DI= deficit irrigation; SDI=severe deficit irrigation

534

535 Figure.2. Representative chromatograms of sorghum grain free polyphenols from
536 genotype MR-Bazley A: under full irrigation regime; B: under deficit irrigation
537 regime; C: under severe deficit irrigation regime.

538

539 Figure.3. Variable projections after principal component analysis (PCA) on six
540 sorghum genotypes a: under full irrigation regime; b: under deficit irrigation regime; c:
541 under severe deficit irrigation regime.

542 Abbreviations: Polyphenols (FCA = free caffeic acid; BCA = bound caffeic acid; TCA = total

543 caffeic acid; FFA = free ferulic acid; BFA = bound ferulic acid; TFA = total ferulic acid;

544 FLUT = free lutedinidin; BLUT = bound lutedinidin; TLUT = total lutedinidin; FAPI = free

545 apigenidin; BAPI = bound apigenidin; TAPI = total apigenidin; FTA = free taxifolin; BTA =

546 bound taxifolin; TTA = total taxifolin; FLU = free luteolin; BLU = bound luteolin; TLU =

547 total luteolin; FNAR = free narigenin; BNAR = bound narigenin; TNAR = total narigenin;

548 FAP = free apigenin; BAP = bound apigenin; TAP = total apigenin); Genotypes (G1 = Liberty;

549 G2 = MR-Bazley; G3 = Alpha; G4 = IS1311C; G5 = IS8237C; G6 = Shawaya Short Black 1)

Table 1. Identification of individual polyphenols in sorghum grains by HPLC-DAD-ESIMS fragmentation and in comparison with respective standards.

Peak No.	Rt ^a (min)	λ_{\max} (nm)	m/z [M - H] ⁻	m/z MS ² (Abundance %)	Tentative identification
1	10.1	275	479	149 (4), 121 (8), 105 (4)	Unknown
2	10.9	280	577	425 (60), 289 (26)	Procyanidin B1
3	11.8	325, 300sh ^b	415	253 (100), 161 (11), 135 (85)	1,2- <i>O</i> -dicaffeoylglycerol isomer
4	12.3	330	353	191 (100), 173 (48), 85 (7)	Chlorogenic acid
5	13.1	326, 298sh	253	179 (1), 161 (100), 135 (65)	2- <i>O</i> -caffeoylglycerol
6	13.5	280, 310	137	109 (35)	Protocatechuic aldehyde
7	14.4	326, 298sh	253	179 (1), 161 (100), 135 (65)	1- <i>O</i> -caffeoylglycerol
8	15.0	297, 322	179	135 (100)	Caffeic acid (std ^c)
9	16.1	280, 490	269	241 (11), 225 (27), 169 (19), 133 (30)	Luteolinidin (std)
10	17.0	290	447	285 (45)	Luteolin hexoside
11	17.7	310	237	163 (20), 145 (50), 119 (31)	2- <i>O-p</i> -coumaroylglycerol
12	18.0	285	433	271 (86);151 (56)	Naringenin hexoside
13	18.3	275, 470	253	225 (10), 209 (70), 179 (40), 117 (65)	Apigeninidin (std)
14	19.1	283	433	287 (100), 151 (59)	Eriodictyol deoxyhexoside
15	19.2	310	163	119 (100)	<i>p</i> -coumaric acid
16	20.8	295, 325	193	178 (42), 134 (100)	Ferulic acid (std)
17	21.6	288	303	285 (100), 217 (9), 177 (18), 125 (35)	Taxifolin (std)
18	28.5	326, 300sh	415	253 (100), 179 (100) , 161 (11), 135 (85)	1,2- <i>O</i> -dicaffeoylglycerol
19	29.4	326, 300sh	415	253 (100), 179 (100) , 161 (11), 135 (85)	1,3- <i>O</i> -dicaffeoylglycerol

20	30.6	287	287	135 (85), 151 (10)	Eriodictyol
21	31.1	252, 347	285	241 (1), 217 (3), 199 (3), 175 (3), 151 (17), 133 (13), 107 (3)	Luteolin (std)
22	33.3	219, 315	399	253 (80), 235 (11), 179 (25), 163 (86), 145 (35), 119 (100), 135 (27)	Coumaroyl-caffeoylglycerol
23	34.2	325, 295sh	429	253 (70), 235 (11), 193 (100), 175 (32), 161 (53), 135 (81)	Feruloyl-caffeoylglycerol
24	35.9	266, 322	269	225 (21), 201 (10), 183 (10), 149 (32), 117 (100)	Apigenin (std)
25	36.3	295	271	177 (10), 151 (50), 119 (20), 107 (10)	Naringenin (std)

^a Rt = Retention time ^b sh = shoulder ^c std= standard

Table 2. Free, bound and total contents of hydroxycinnamic acids ($\mu\text{g/g}$ dry basis) of six genotypes of sorghum grain grown under three irrigation treatments.

		Genotype					
		Liberty	MR-Bazley	Alpha	IS1311C	IS8237C	Shawaya Short Black 1
Caffeic acid							
Free	FI	7.18 \pm 0.29 ^{eB}	5.04 \pm 0.10 ^{bA}	12.55 \pm 0.29 ^{fA}	4.51 \pm 0.38 ^{aA}	5.54 \pm 0.26 ^{cA}	6.49 \pm 0.53 ^{dC}
	DI	10.64 \pm 0.82 ^{cC}	9.84 \pm 0.21 ^{bC}	19.76 \pm 0.67 ^{eC}	9.34 \pm 0.62 ^{bB}	14.97 \pm 0.25 ^{dB}	5.08 \pm 0.17 ^{aA}
	SDI	6.44 \pm 0.18 ^{bA}	6.78 \pm 0.32 ^{bB}	13.00 \pm 0.18 ^{dB}	11.38 \pm 0.48 ^{cC}	No data	5.44 \pm 0.08 ^{aB}
Bound	FI	5.76 \pm 0.38 ^{cA}	9.54 \pm 0.11 ^{dB}	10.47 \pm 0.34 ^{eC}	4.63 \pm 0.10 ^{bA}	4.18 \pm 0.09 ^{aB}	5.03 \pm 0.21 ^{cA}
	DI	9.97 \pm 0.53 ^{dC}	7.20 \pm 0.23 ^{cA}	7.56 \pm 0.10 ^{cB}	10.38 \pm 1.17 ^{dB}	3.66 \pm 0.12 ^{aA}	5.13 \pm 0.10 ^{bA}
	SDI	6.98 \pm 1.03 ^{aB}	14.71 \pm 0.88 ^{cC}	6.97 \pm 0.28 ^{aA}	15.59 \pm 0.88 ^{cC}	No data	10.05 \pm 0.49 ^{bB}
Total	FI	12.94 \pm 0.68 ^{dA}	14.58 \pm 0.01 ^{eA}	23.21 \pm 0.77 ^{fB}	9.14 \pm 0.28 ^{aA}	9.72 \pm 0.16 ^{bA}	11.55 \pm 0.76 ^{cA}
	DI	20.61 \pm 1.40 ^{dB}	17.04 \pm 0.43 ^{bB}	27.49 \pm 0.70 ^{eC}	19.73 \pm 1.79 ^{dB}	18.62 \pm 0.16 ^{cB}	10.13 \pm 0.34 ^{aA}
	SDI	13.42 \pm 1.21 ^{aA}	21.48 \pm 0.57 ^{dC}	19.98 \pm 0.05 ^{cA}	26.97 \pm 1.90 ^{eC}	No data	16.11 \pm 0.70 ^{bB}
Ferulic acid							
Free	FI	1.18 \pm 0.20 ^{aA}	1.98 \pm 0.13 ^{dA}	1.58 \pm 0.21 ^{bA}	2.03 \pm 0.01 ^{dA}	1.55 \pm 0.12 ^{bA}	1.65 \pm 0.10 ^{aA}
	DI	1.97 \pm 0.11 ^{aC}	2.87 \pm 0.01 ^{cC}	2.78 \pm 0.16 ^{cC}	4.83 \pm 0.20 ^{dC}	2.34 \pm 0.24 ^{bB}	2.36 \pm 0.07 ^{bB}
	SDI	1.82 \pm 0.06 ^{aB}	2.31 \pm 0.13 ^{bB}	2.46 \pm 0.03 ^{cB}	2.48 \pm 0.22 ^{cB}	No data	2.97 \pm 0.21 ^{dC}
Bound	FI	45.87 \pm 2.14 ^{cB}	82.60 \pm 3.57 ^{eB}	60.47 \pm 1.70 ^{dB}	38.11 \pm 5.68 ^{bA}	28.87 \pm 0.75 ^{aA}	46.91 \pm 2.29 ^{cA}
	DI	50.97 \pm 1.78 ^{bC}	78.75 \pm 2.10 ^{dA}	40.56 \pm 0.77 ^{aA}	114.82 \pm 3.59 ^{eC}	52.69 \pm 2.23 ^{bcB}	55.18 \pm 0.69 ^{cB}
	SDI	35.51 \pm 3.15 ^{aA}	100.23 \pm 1.20 ^{eC}	42.66 \pm 0.59 ^{bA}	61.01 \pm 1.67 ^{cB}	No data	66.85 \pm 2.49 ^{dC}
Total	FI	47.05 \pm 2.33 ^{cB}	84.58 \pm 3.43 ^{eB}	62.48 \pm 1.82 ^{dC}	40.14 \pm 5.69 ^{bA}	30.43 \pm 0.83 ^{aA}	47.87 \pm 2.94 ^{cA}
	DI	52.94 \pm 1.89 ^{bC}	81.62 \pm 2.11 ^{dA}	43.17 \pm 0.75 ^{aA}	119.65 \pm 3.79 ^{eC}	55.03 \pm 2.47 ^{bcB}	57.72 \pm 0.76 ^{cB}
	SDI	37.33 \pm 3.22 ^{aA}	102.54 \pm 1.37 ^{eC}	45.26 \pm 0.58 ^{bB}	63.49 \pm 1.45 ^{cB}	No data	70.61 \pm 3.32 ^{dC}

^{a-f} Values with different superscripts in the same row are significantly different ($P \leq 0.05$). ^{A, B, C} Values with different superscripts in the same column in the same dependent variable are significantly different ($P \leq 0.05$).

Table 3. Free, bound and total individual flavonoid content ($\mu\text{g} / \text{g}$, dry basis) of six genotypes of sorghum grain grown in three irrigation treatments.

Flavonoids		Genotypes						
		Liberty	MR-Bazley	Alpha	IS1311C	IS8237C	Shawaya Short Black 1	
<i>3-Deoxyanthocyanidins</i>								
Luteolinidin	Free	FI	nd	0.26±0.04 ^{aB}	0.48±0.09 ^{bA}	1.58±0.08 ^{cA}	4.81±0.18 ^{dA}	13.44±0.76 ^{eB}
		DI	nd	1.29±0.11 ^{aC}	2.30±0.04 ^{bC}	2.42±0.44 ^{cB}	6.90±0.13 ^{dB}	18.25±0.98 ^{eC}
		SDI	nd	0.04±0.01 ^{aA}	1.61±0.20 ^{dB}	1.49±0.20 ^{cA}	No data	1.34±0.10 ^{bA}
	Bound	FI	nd	3.31±0.16 ^{dB}	0.71±0.05 ^{aA}	0.84±0.06 ^{bC}	2.15±0.04 ^{cA}	11.72±0.73 ^{eB}
		DI	nd	8.91±0.57 ^{dC}	3.11±0.24 ^{bB}	0.66±0.15 ^{aB}	4.41±0.10 ^{cB}	11.60±1.40 ^{eB}
		SDI	nd	2.86±0.13 ^{cA}	0.68±0.10 ^{bA}	0.30±0.02 ^{aA}	No data	0.34±0.07 ^{aA}
	Total	FI	nd	3.56±0.20 ^{cA}	1.20±0.05 ^{aA}	2.41±0.01 ^{bB}	5.95±0.21 ^{dA}	25.16±0.04 ^{eB}
		DI	nd	10.21±0.23 ^{bB}	5.41±0.20 ^{cC}	3.08±0.59 ^{aC}	7.36±0.21 ^{dB}	29.98±0.51 ^{eC}
		SDI	nd	2.90±0.55 ^{cC}	2.33±0.12 ^{bB}	1.79±0.22 ^{aA}	No data	1.73±0.21 ^{aA}
Apigeninidin	Free	FI	nd	1.02±0.11 ^{bB}	0.68±0.07 ^{aB}	1.63±0.11 ^{cB}	5.17±0.09 ^{dA}	16.42±0.35 ^{eB}
		DI	nd	3.67±0.18 ^{bC}	0.39±0.02 ^{aA}	3.96±0.30 ^{bC}	8.17±0.15 ^{cB}	18.89±0.28 ^{dC}
		SDI	nd	0.08±0.02 ^{aA}	1.41±0.14 ^{cC}	1.37±0.16 ^{cA}	No data	1.18±0.08 ^{bA}
	Bound	FI	nd	10.49±0.37 ^{eC}	4.42±0.30 ^{cB}	1.71±0.39 ^{aA}	3.09±0.03 ^{bB}	9.04±0.35 ^{dB}
		DI	nd	9.62±0.49 ^{bB}	15.66±0.44 ^{cC}	19.62±0.93 ^{dC}	1.29±0.06 ^{aA}	9.85±0.43 ^{bC}
		SDI	nd	2.53±0.13 ^{bA}	3.85±0.24 ^{cA}	9.70±1.74 ^{dB}	No data	1.26±0.10 ^{aA}
	Total	FI	nd	11.51±0.48 ^{dB}	5.22±0.46 ^{bA}	3.34±0.28 ^{aA}	8.25±0.13 ^{cA}	25.26±0.86 ^{eB}
		DI	nd	13.28±0.31 ^{bC}	16.05±0.41 ^{cB}	23.57±1.23 ^{dC}	9.47±0.08 ^{aB}	28.95±0.88 ^{eC}

	SDI	nd	2.61±0.11 ^{aA}	5.19±0.24 ^{bA}	11.07±1.89 ^{cB}	No data	2.49±0.21 ^{aA}	
<i>Flavones</i>								
Free	FI	0.38±0.06 ^{aA}	3.48±0.10 ^{cA}	1.98±0.08 ^{bA}	8.20±0.04 ^{dB}	15.11±0.03 ^{eA}	12.59±0.54 ^{eB}	
	DI	0.74±0.13 ^{aC}	6.62±0.04 ^{cC}	3.37±0.16 ^{bB}	10.79±0.13 ^{dC}	19.14±0.09 ^{eB}	19.74±1.23 ^{eC}	
	SDI	0.67±0.04 ^{aB}	4.08±0.02 ^{cB}	1.94±0.13 ^{bA}	7.33±0.08 ^{eA}	No data	5.59±0.08 ^{dA}	
Luteolin	Bound	FI	1.72±0.08 ^{bB}	3.58±0.30 ^{cB}	0.38±0.02 ^{aA}	0.40±0.08 ^{aA}	4.13±0.13 ^{dA}	3.23±0.23 ^{cB}
	DI	3.83±0.22 ^{cC}	5.53±0.09 ^{eC}	0.55±0.01 ^{aB}	1.35±0.13 ^{bC}	3.61±0.09 ^{cB}	4.37±0.25 ^{dC}	
	SDI	1.41±0.12 ^{cA}	1.71±0.19 ^{cA}	0.86±0.12 ^{aC}	1.09±0.06 ^{bB}	No data	1.49±0.32 ^{cA}	
Total	FI	2.10±0.02 ^{aA}	6.06±0.40 ^{cB}	2.33±0.12 ^{bA}	8.60±0.13 ^{dA}	19.24±0.10 ^{eA}	15.82±0.30 ^{eB}	
	DI	4.57±0.35 ^{bB}	12.15±0.13 ^{cC}	3.92±0.18 ^{aC}	12.14±0.26 ^{cB}	22.75±0.17 ^{dB}	24.11±0.98 ^{eC}	
	SDI	2.07±0.16 ^{aA}	5.78±0.17 ^{cA}	2.79±0.01 ^{bB}	8.42±0.14 ^{eA}	No data	7.07±0.40 ^{dA}	
Apigenin	Free	FI	nd	2.16±0.24 ^{bA}	nd	2.28±0.26 ^{bA}	4.59±0.11 ^{cA}	0.98±0.35 ^{aA}
	DI	nd	4.97±0.35 ^{bB}	nd	6.04±0.54 ^{cC}	6.70±0.19 ^{dB}	1.94±0.39 ^{aB}	
	SDI	nd	2.10±0.31 ^{bA}	nd	4.61±0.17 ^{cB}	No data	0.92±0.13 ^{aA}	
Bound	FI	nd	8.97±0.08 ^{dB}	nd	3.39±0.24 ^{bA}	7.48±0.22 ^{cA}	1.83±0.51 ^{aC}	
	DI	nd	23.14±1.34 ^{dC}	nd	5.71±0.46 ^{bC}	13.60±0.37 ^{cB}	1.08±0.40 ^{aB}	
	SDI	nd	7.05±0.35 ^{cA}	nd	4.03±0.08 ^{bB}	No data	0.91±0.36 ^{aA}	
Total	FI	nd	11.13±0.32 ^{cB}	nd	5.67±0.02 ^{bA}	12.07±0.33 ^{cA}	2.81±0.88 ^{aB}	
	DI	nd	28.10±1.73 ^{dC}	nd	11.74±1.00 ^{bC}	20.30±0.37 ^{cB}	3.02±0.78 ^{aC}	
	SDI	nd	9.15±0.04 ^{cA}	nd	8.64±0.05 ^{bB}	No data	1.83±0.49 ^{aA}	

<i>Dihydroflavonol</i>									
Taxifolin	Free	FI	nd	6.93±0.08 ^{bb}	9.35±0.24 ^{dC}	5.43±0.06 ^{aA}	8.19±0.30 ^{cA}	14.56±0.34 ^{eB}	
		DI	nd	11.47±0.31 ^{bc}	8.20±0.21 ^{aB}	28.64±1.03 ^{eC}	14.93±0.27 ^{cB}	16.02±0.57 ^{dC}	
		SDI	nd	0.31±0.04 ^{aA}	3.01±0.11 ^{bA}	11.18±0.36 ^{dB}	No data	8.86±0.25 ^{cA}	
	Bound	FI	nd	2.41±0.40 ^{aA}	6.12±0.14 ^{dA}	4.53±0.47 ^{bA}	5.11±0.29 ^{cA}	10.92±1.14 ^{eB}	
		DI	nd	9.58±0.69 ^{aC}	9.73±0.20 ^{aC}	12.33±1.34 ^{bc}	16.48±0.29 ^{cB}	18.50±0.30 ^{dC}	
		SDI	nd	6.53±0.13 ^{bb}	8.51±0.54 ^{cb}	10.24±0.59 ^{bb}	No data	4.67±0.43 ^{aA}	
	Total	FI	nd	9.34±0.32 ^{aB}	15.50±0.13 ^{cb}	9.96±0.53 ^{aA}	13.30±0.56 ^{bA}	25.48±0.82 ^{dB}	
		DI	nd	21.05±1.00 ^{bc}	17.93±0.01 ^{aC}	40.97±2.34 ^{dC}	31.41±0.02 ^{cb}	34.52±0.26 ^{cC}	
		SDI	nd	6.83±0.17 ^{aA}	11.34±0.80 ^{bA}	21.41±0.23 ^{dB}	No data	13.53±0.67 ^{cA}	
<i>Flavanone</i>									
Naringenin	Free	FI	nd	1.70±0.06 ^{aB}	15.66±0.05 ^{dA}	5.80±0.42 ^{bA}	11.32±0.44 ^{cA}	23.39±0.80 ^{eB}	
		DI	nd	2.92±0.07 ^{aC}	31.88±0.11 ^{eC}	12.47±0.31 ^{bc}	13.19±0.13 ^{cb}	30.60±2.23 ^{dC}	
		SDI	nd	1.15±0.05 ^{aA}	22.51±0.07 ^{dB}	7.69±0.87 ^{bb}	No data	17.63±1.55 ^{cA}	
	Bound	FI	nd	3.48±0.25 ^{aB}	5.54±0.19 ^{bA}	6.11±0.28 ^{cb}	8.04±0.10 ^{dA}	16.64±1.18 ^{eB}	
		DI	nd	4.07±0.22 ^{aC}	8.88±0.23 ^{bc}	9.09±0.31 ^{cC}	10.47±0.21 ^{dB}	17.87±0.43 ^{eC}	
		SDI	nd	2.74±0.07 ^{aA}	6.96±0.15 ^{cb}	4.58±0.45 ^{bA}	No data	12.50±2.11 ^{dA}	
	Total	FI	nd	5.18±0.19 ^{aB}	21.14±0.29 ^{cA}	11.91±0.46 ^{bA}	19.36±1.13 ^{cA}	40.02±1.62 ^{dB}	
		DI	nd	6.99±0.27 ^{aC}	40.76±0.31 ^{dC}	21.56±0.72 ^{bb}	23.65±0.07 ^{cb}	48.46±2.66 ^{eC}	
		SDI	nd	3.89±0.12 ^{aA}	29.49±0.10 ^{cb}	12.27±0.66 ^{bA}	No data	30.13±1.86 ^{cA}	

^{a-e} Values with different superscripts in the same row are significantly different ($p \leq 0.05$). ^{A, B, C} Values with different superscripts in the same column in the same

dependent variable are significantly different ($p \leq 0.05$). nd= not detected.

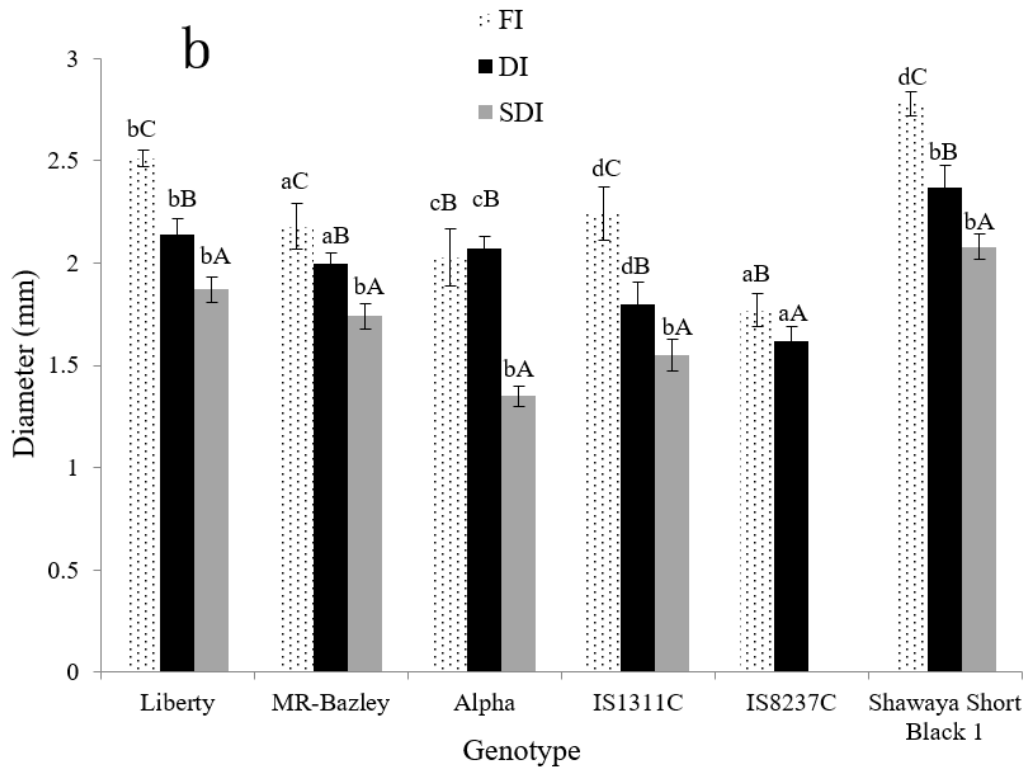
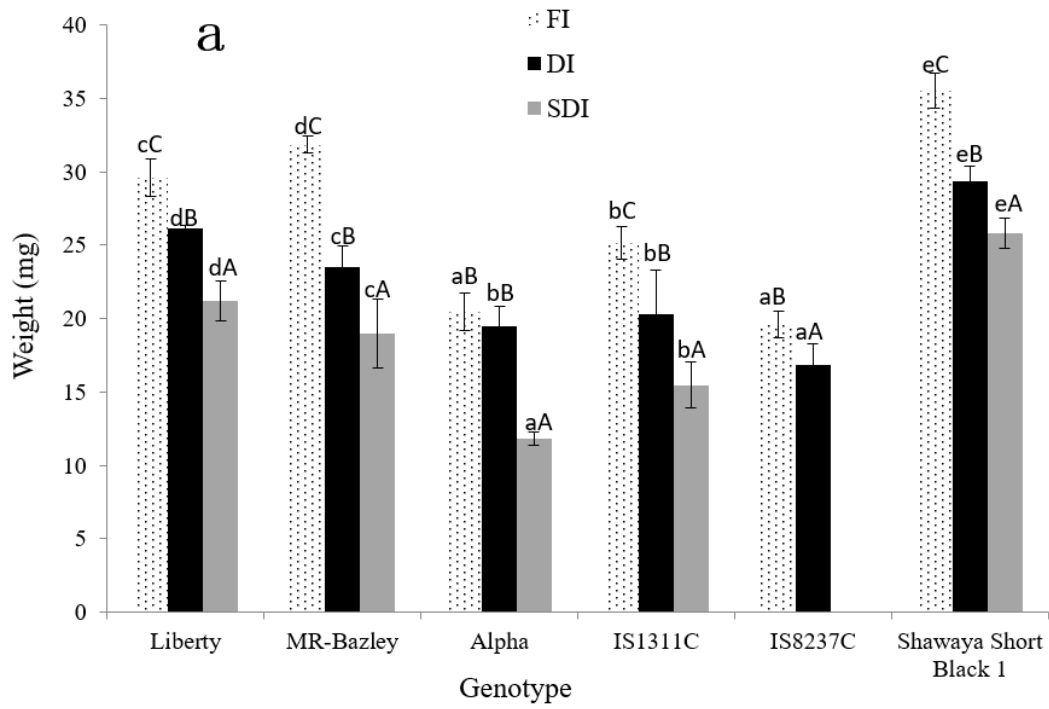


Figure 1

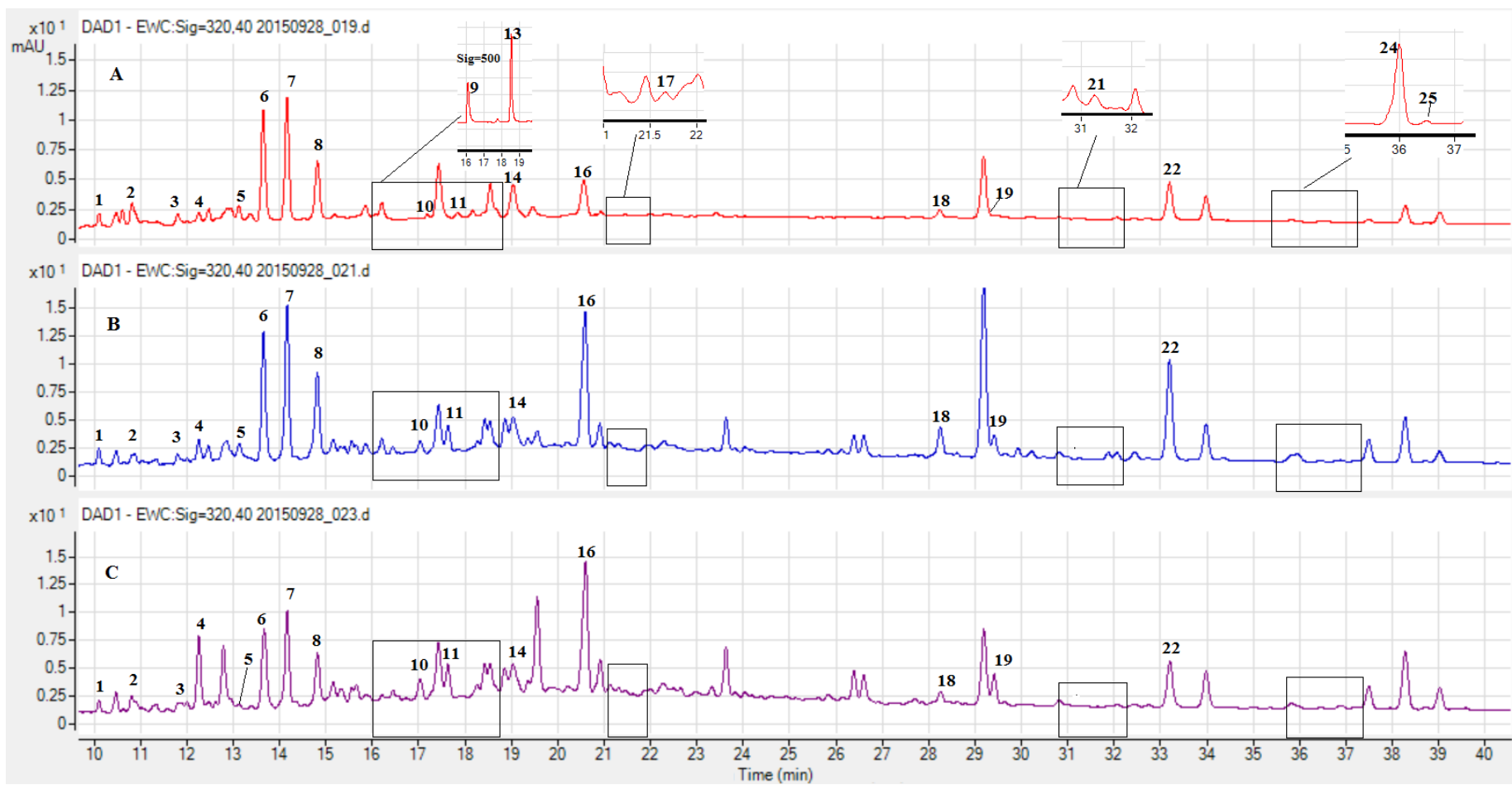
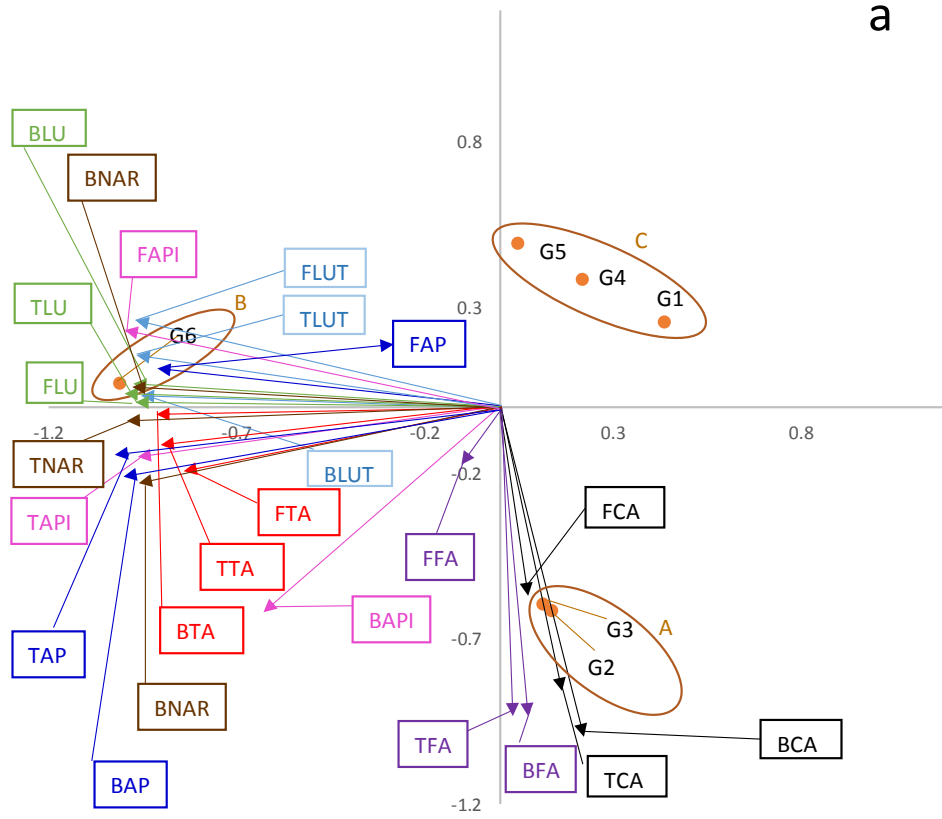
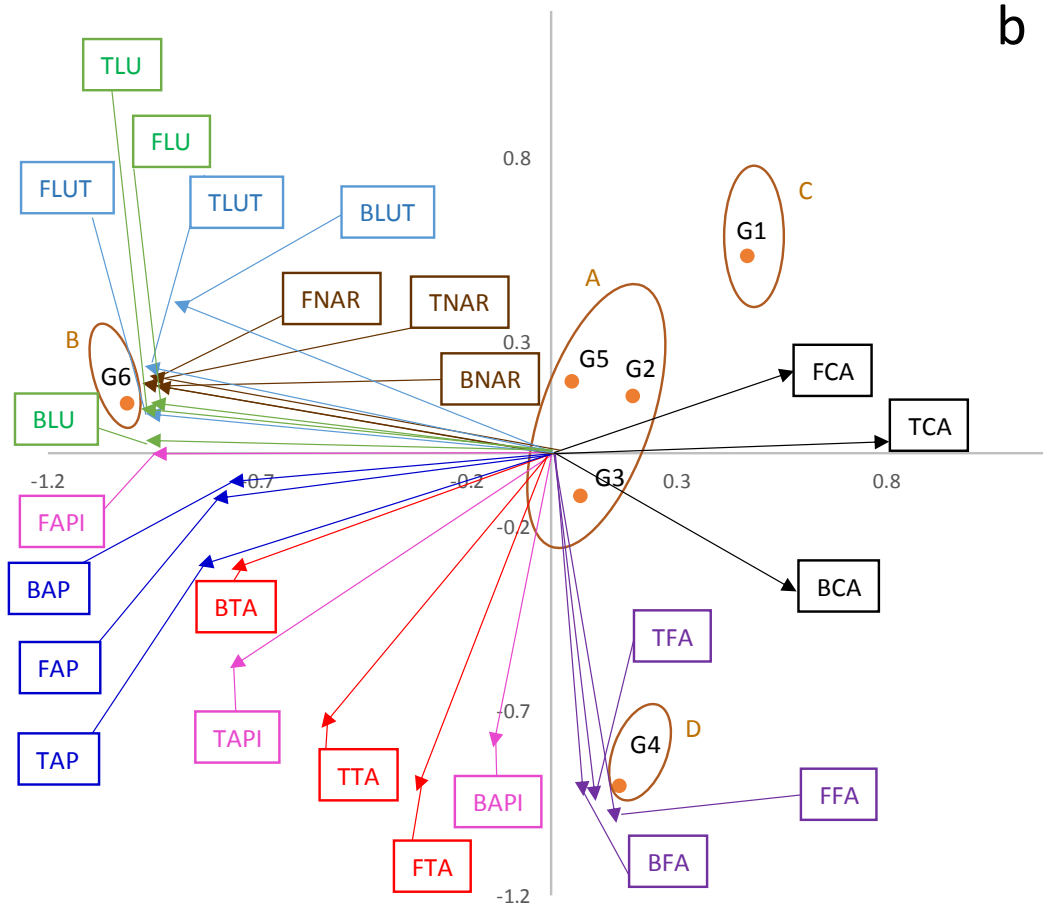


Figure 2

a



b



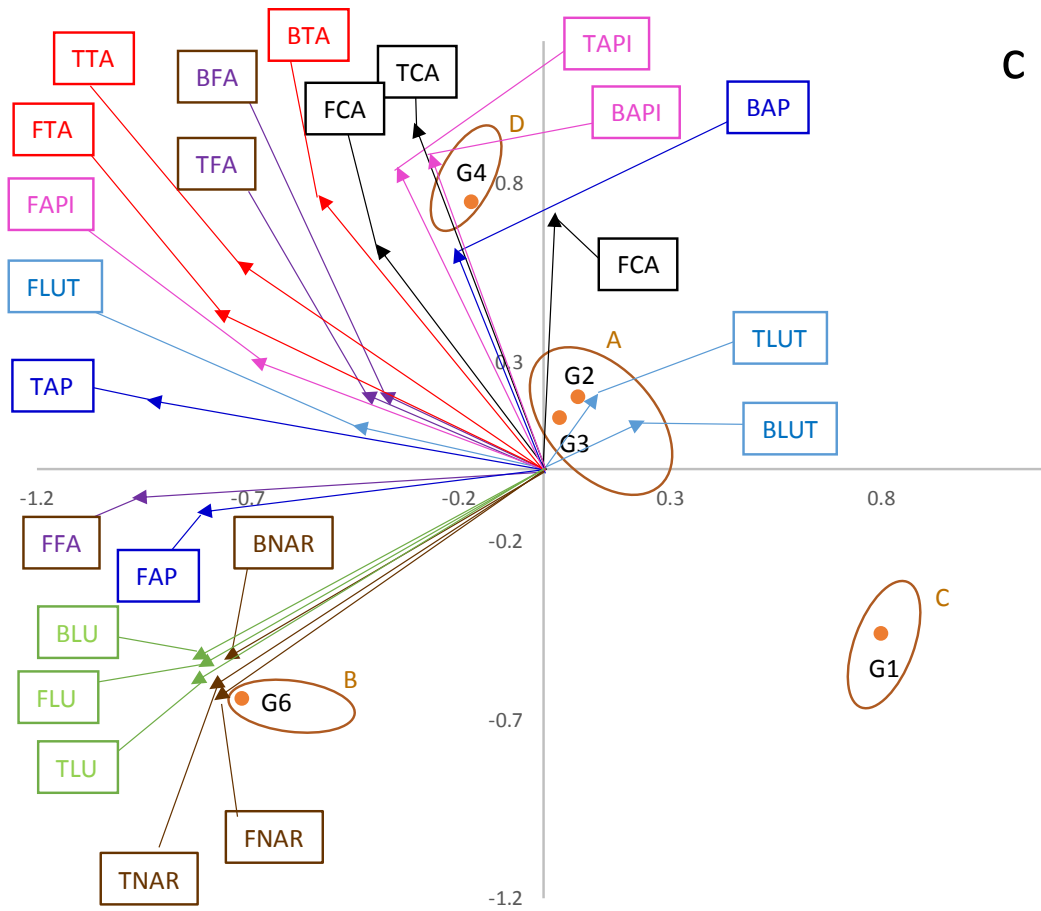


Figure 3