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Antioxidant capacity and vitamin E in barley: effect of genotype and storage

Thi Thu Dung Do, Daniel Cozzolino, Beverly Muhlhausler, Amanda Box, Amanda J. Able*

School of Agriculture, Food & Wine, University of Adelaide Waite Research Institute, PMB 1 Glen Osmond, SA 5064, South Australia, Australia

Authors' email addresses

Thi Thu Dung Do: thithudung.do@adelaide.edu.au

Daniel Cozzolino: d.cozzolino@adelaide.edu.au

Beverly Muhlhausler: beverly.muhlhausler@adelaide.edu.au

Amanda Box: amanda.box@adelaide.edu.au

Amanda Able: amanda.able@adelaide.edu.au

* Corresponding author:

Assoc. Prof. Amanda J. Able

School of Agriculture, Food and Wine, Waite Campus

The University of Adelaide, PMB 1, Glen Osmond, SA 5064, South Australia, Australia

Tel: +61 8 8313 7245

Fax: +61 8 8313 7109

Email: amanda.able@adelaide.edu.au

Running title:

Antioxidant capacity and vitamin E in barley genotypes.

26 **Abstract**

27 Antioxidants, including vitamin E, may have a positive effect on human health and prolong
28 storage of food items. Vitamin E content and antioxidant capacity were measured in 25
29 barley genotypes before and after 4 months storage at 10°C using high performance liquid
30 chromatography (HPLC) and ability to scavenge DPPH radicals, respectively. As expected,
31 α-tocotrienol (α-T3) and α-tocopherol (α-T) were the predominant tocol isomers. Vitamin E
32 content and antioxidant capacity varied significantly among genotypes. Vitamin E ranged
33 from 8.5 to 30.8 μg/g dry weight (DW) while ascorbic acid equivalent antioxidant capacity
34 (AEAC) varied from 57.2 to 158.1 mg AEAC/100 g fresh weight (FW). Generally, lower
35 vitamin E content or antioxidant capacity was observed in hulless or coloured genotypes.
36 Results suggest some genotypes are potential candidates for breeding of barley cultivars with
37 high vitamin E content or antioxidant capacity at harvest and even after storage.

38

39 **Keywords**: Barley, genotypes, vitamin E, antioxidant, storage.

40

41

42 **1. Introduction**

43 Barley (*Hordeum vulgare* L.) has historically been used for malt and livestock feed. 44 Recently, however, there has been growing interest in the potential use of barley in human 45 foods largely due to its high content of fibre, β-glucan and antioxidants (Ehrenbergerova, 46 Belcrediova, Havlova, Pryma, Vaculova, & Vejrazka, 2006). Barley is likely to be a good 47 dietary source of antioxidants not only because of its high antioxidant capacity but also 48 because of the relatively larger amounts consumed in typical diets compared to fruit and 49 vegetables (Kim, Hyun, Kim, Park, Kim, Kim et al., 2007). Antioxidants are crucial in 50 maintaining the health of tissues and organs because of their ability to slow tissue damage by 51 preventing the formation of free radicals, scavenging them, or by promoting their 52 decomposition (Young & Woodside, 2001). Antioxidant capacity has been reported to be 53 higher in coloured than white grains, including rice (Htwe, Srilaong, Tanprasert, 54 Tongchitpakdee, Kanlayanarat, & Uthairatanakij, 2010) and barley germplasm (Kim et al., 55 2007). In addition, the antioxidants concentrate in the outer layers of the grain (Peterson, 56 1994). Thus, removal of these layers (the hull, aleurone and germ) in covered barley by the 57 process of pearling, used to make flour whiter, significantly reduces antioxidant capacity 58 (Bhatty, 1999). Hulless genotypes, therefore, may have an enriched antioxidant capacity, 59 since they can be used without the requirement for pearling. The different genotypes across 60 coloured and white barley; covered and hulless barley; food, malting and feed barley are also 61 likely to differ in antioxidant capacity.

62 Antioxidants are generally accepted as including vitamin E (tocotrienols and 63 tocopherols), ascorbic acid (vitamin C), enzymes (catalase, glutathione peroxidase and 64 superoxide dismutase), phenolic compounds, and carotenoids (Goupy, Hugues, Boivin, & 65 Amiot, 1999). Vitamin E is a lipid phase chain-breaking antioxidant that appears effective in 66 improving health outcomes in clinical trials with diminished risk of cancer and cardiovascular

disease, especially in smokers (Reboul, Richelle, Perrot, Desmoulins-Malezet, Pirisi, & Borel, 2006), although benefits have not been reported in all studies (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007). Vitamin E has eight isomers: α-, β-, γ-, δ-tocopherol (T) and α-, β-, γ-, δ-tocotrienol (T3). Tocopherol and tocotrienol are also called tocols. Ball (2006) reported the descending order of their antioxidant capacity to be α-, β-, γ- and δ-tocols. Sheppard et al., (1993) however found that among T and T3, the descending order of antioxidant activity was: α-T, β-T, α-T3, γ-T, β-T3, and δ-T (γ-T3 and δ-T3 had no function). However, some studies reported that α-T3 is a more effective antioxidant than α-T (Packer 1995).

Among different cereals, vitamin E has been reported to be higher in barley compared to other grains (Panfili, Fratianni, & Irano, 2003). Therefore, barley has potential for use as a functional food providing vitamin E. However, the reported content of vitamin E in barley differs between studies, for example 51.6 µg/g DW in Ehrenbergerova, Belcrediova, Havlova, et al. (2006), 59.0 µg/g DW in Bhatty (1999) and 69.1 µg/g DW in Panfili (2008). The contribution to vitamin E content of the eight isomers in these studies was also different. The discrepancies between studies could be due, at least in part, to the fact that different genotypes were studied, as different genotypes may contain different amounts of vitamin E. These studies also differed in the extraction methods used, that is, whether saponification was used and which solvent. Methods using saponification and hexane as a solvent are currently regarded as best for tocol extraction in cereals because esterification is prevented and recovery is greatest (Panfili et al., 2003).

Another issue to be considered is storage. Antioxidants and vitamin E, in particular, can be easily destroyed by light, water and heat (Wang, Xue, Newman, & Newman, 1993). Newly harvested barley will often be in storage for between 4 and 18 months before processing (Idaho Barley Commission, 2011). Even though barley is usually stored in silos

92 with cooling aeration systems at less than 15°C and ideally to 10°C (Viljoen, 2001); storage
93 may have an effect on vitamin E content and antioxidant capacity. Indeed, storage at 25, 27
94 or 35°C led to a significant loss of vitamin E content (Liu & Moreau, 2008; Wang et al.,
95 1993). However, research has not been published on the influence of storage on both
96 antioxidant capacity and vitamin E content in barley at the usual temperatures used by
97 industry.

98 This study focused not only on the antioxidant capacity of barley genotypes but also
99 on the content of vitamin E, which is often claimed to be an important antioxidant compound.
100 The objectives of this study were to evaluate these components in a range of barley genotypes
101 and the impact on them of storage under conditions similar to those used in industry. Barley
102 genotypes were chosen based on their grain colour, whether hulless or not, and their use in
103 food, malt or animal feed.

104

105 **2. Materials and Methods**

106 *2.1. Materials*

107 Materials in this study, provided by the University of Adelaide Barley Breeding
108 Program, were 25 common food, malting and animal feed genotypes including hulless and
109 covered, coloured and white barley (which can be categorised by genotype as Food,
110 Coloured, Hulless: Jet, Sumire mochi; Food, White, Hulless: Finniss, Macumba; Food,
111 Colored, Covered: Tadmor; Food, White, Covered: Adagio, Er/Apm; Malting, White,
112 Covered: Flagship, WI2585, Vlamingh, Amaji Nijo, Harrington, ND24260-1, Commander,
113 Alexis, Dhow, Sloop, Buloke; Feed, Coloured, Covered: ICARDA 16, ICARDA 19,
114 ICARDA 26, ICARDA 35, ICARDA 39; Feed, White, Covered: Fleet, Chebec)
115 (Supplementary Information, Table S1). All 25 genotypes were grown from June 2011 to
116 December 2011 as a single plot in a complete randomised design at Charlick Experimental

117 Research Station, Strathalbyn, South Australia (35°19'46.26" S, 138°52'42.39" E). After
118 harvesting, the grains were screened using a 2.5 mm slotted ISO 5223 sieve. The grains were
119 kept at -20°C until their moisture content, antioxidant capacity and vitamin E content were
120 analysed for at least three individual biological replicates.
121
122 *2.2. Storage*
123 After harvesting, 1 kg of barley grains from all genotypes except Jet, ICARDA 26 and
124 Buloke, which were not available to plant, was cleaned and placed in a 300 x 200 x 100 mm
125 plastic box with lid. Beakers of silica gel (50 mL) were placed in the containers to ensure
126 humidity was maintained, as confirmed by humidity meters. The grain boxes were stored at
127 10°C in the dark for 4 months, as is common industrial practice (Idaho Barley Commission,
128 2011). The moisture content, vitamin E content and antioxidant capacity were analysed for at
129 least three individual biological replicates for each genotype before and after 4 months of
130 storage.
131
132 *2.3. Grinding*
133 Barley grain (10 g) for each genotype was ground to a fine powder using an IKA Mill
134 (Germany) with running water to avoid heat increases during the milling.
135
136 *2.4. Moisture content*
137 The moisture content of barley flour was determined in triplicate according to the
138 procedure described in American Association of Cereal Chemists-AACC (2000) Method No.
139 44-15A. Two grams of sample was weighed into a pre-weighed dish and dried in an air
140 forced oven at a temperature of 105±5°C until the weight was constant.
141

142 *2.5. Determination of Vitamin E content*

143 *2.5.1. Vitamin E extraction*

144 To extract tocols from barley flour and avoid degradation of the isomers, an optimised
145 method using hot saponification was adapted from that utilised by Lampi, Ryynanen, Salo-
146 Vaananen, Ollilainen and Piironen (2004). Flour (0.1 g) was added to a solution of 1 mL
147 100% (v/v) ethanol, 0.4 mL water and 20 mg ascorbic acid in a 15 mL Pyrex glass tube with
148 a Teflon screw cap. After addition of 100 μL 10.7 M potassium hydroxide solution and
149 thorough mixing, the tube was capped and transferred to a boiling water bath for 25 min.
150 During saponification, the sample was mixed every 10 min to improve the hydrolysis. The
151 tube was cooled in an ice water bath for 10 min and 0.5 mL 50% (v/v) ethanol was added. To
152 extract tocols and other unsaponifiable lipids, three portions (each 2 mL) of n-hexane:ethyl
153 acetate (8:2, v/v) were used. After shaking samples and solvent for 10 min and separation of
154 the phases, the upper organic layers were collected with a disposable glass pipette and placed
155 into a new glass test tube. This process was repeated three times with n-hexane:ethyl acetate
156 (8:2, v/v), and the extract was then dried using stream nitrogen. The residue was dissolved in
157 1 mL n-hexane and filtered through a 0.45 μm syringe filter before transfer to a 12x32
158 GRACE glass HPLC (high-performance liquid chromatography) vial with an amber screw
159 cap for analysis.

160

161 *2.5.2. HPLC analysis*

162 The tocols were quantified according to the method reported by Lampi et al. (2004)
163 with some modification. Tocols were separated by a normal phase HPLC using a GRACE
164 Altima HP Silica 150 x 3 mm, 3 micron column and quantified with a fluorescence detector
165 (NP-HPLC-FLD) with an excitation wavelength of 290 nm and an emission wavelength of

166 325 nm. The mobile phase was 1,4-dioxane/n-hexane (2:98, v/v) at a flow rate of 1 mL/min.
167 Separation of tocols was based on isocratic elution (Lampi et al., 2004).

168 The quantity of the individual vitamin E isomers in samples was calculated by the use
169 of calibration curves for all standard isomers. Tocopherol (T) standards (α-, β-, γ-, δ-T) and
170 tocotrienol (T3) standards (α-, β-, γ-, δ-T3) were purchased from Cayman Chemical, USA,
171 while n-hexane, ethyl acetate, ascorbic acid and DPPH (2, 2-diphenyl-1-picrylhydrazyl) were
172 from Chem-Supply Pty Ltd, Australia. Standard stock solutions were prepared to a
173 concentration of 500 µg/mL in hexane. Calibration curves for all isomers were prepared over
174 the concentration range of 1.0 to 25.0 µg/mL using GenStat 14 (Lawes Agricultural Trust;
175 VSN International, Ltd., Hemel Hempstead, UK).

176 Barley extractions and standard isomers were injected into the HPLC machine
177 separately. Identification of peaks in barley samples was made based on the retention time
178 when compared with standard peaks. Curves between standard peaks and standard contents
179 were used to calculate contents of isomers in barley samples.

180 The vitamin E content, expressed in mg of α-tocopherol-equivalents (TE), was
181 calculated according to Mclaughlin and Weihrauch (1979) using biological activities of 1.0
182 for α-T, 0.3 for α-T3, 0.4 for β-T, 0.05 for β-T3, 0.1 for γ-T, 0.01 for γ-T3 and 0.01 for δ-T.
183 (α-TE = α-T*1.0 + α-T3*0.3 + β-T*0.4 + β-T3*0.05 + γ-T*0.1 + γ-T3*0.01 + δ-T*0.01).

184

185 *2.6. Determination of antioxidant capacity*

186 *2.6.1. Ethanol extraction of antioxidants in whole grain*

187 Barley flour (1 g) was extracted with 20 mL 80% ethanol at 45ºC in a flask placed in
188 a 200 rpm shaking water bath for 4 h under dark conditions. Vacuum filtration was then used
189 to separate the supernatant, which was stored in the dark at -20ºC and analysed within 24 h
190 using the DPPH free radical method (Omwamba & Hu, 2009).

191

192 *2.6.2. DPPH free radical method*

193 Barley flour extract (0.1 mL) was added to 2.9 mL of DPPH (112 μM). After mixing,
194 the sample was allowed to stand at 23°C in the dark for 20 min. Reduction in absorbance was
195 measured at 517 nm after 20 min using a spectrophotometer (UV/VIS SP 8001, Metertech,
196 Taiwan). Antioxidant capacity was then determined using a standard curve for ascorbic acid
197 and prediction models provided by GenStat 14 and expressed as mg ascorbic acid equivalent
198 antioxidant capacity per 100 g of fresh weight of grain (mg AEAC/100 g FW).

199

200 *2.6.3. Determination of antioxidant capacity of vitamin E*

201 The vitamin E was extracted as described previously (2.6.1). After extraction and
202 drying using stream nitrogen, 1 mL n-hexane was replaced by 1 mL of 100% (v/v) ethanol to
203 dissolve the residue. The antioxidant capacity of the vitamin E extracts was then determined
204 using the DPPH free radical method (2.6.2).

205

206 *2.7. Mid-infrared (MIR) measurement*

207 To determine whether there were any identifiable biochemical differences between
208 samples that differed in vitamin E or antioxidant capacity after storage, MIR was applied.
209 Barley flour, from fresh and stored samples, was scanned using a platinum diamond ATR
210 single reflection sampling module cell mounted in a Bruker Alpha instrument (Bruker Optics
211 GmbH, Ettlingen, Germany) and spectra were recorded on OPUS software version 7.0
212 provided by Bruker Optics (average of 64 scans at a resolution of 8 cm^{-1}, between 4000 and
213 375 cm^{-1}) (Cozzolino, Roumeliotis, & Eglinton, 2013). The samples were held against the
214 ATR crystal using the pressure applicator or sample clamp mechanism supplied by the

215 instrument manufacturer to ensure that the same and constant pressure was applied for all
216 replicates. Air was used as reference background spectra.
217

218 *2.8. Statistical analysis*

219 One-way and two-way Analysis of Variance (ANOVA) was performed using GenStat
220 14 to determine the differences between means using the Least Significant Difference (LSD)
221 at $P<0.05$. Spectra were exported from the OPUS software into The Unscrambler X software
222 (version X, CAMO ASA, Oslo, Norway) for chemometric analysis, data processed using the
223 second derivative Savitzky-Golay and then Principal Component Analysis (PCA) was used to
224 interpret the changes in the MIR spectra of the samples related to storage, as per Cozzolino et
225 al. (2013).
226

227 **3. Results**

228 *3.1. Tocol and antioxidant capacity are genotype-dependent in barley grain*

229 The isomers were easily distinguishable in the order of α-T, α-T3, β-T, γ-T, β-T3, γ-
230 T3 (Supplementary Information, Fig. S1). The retention times (t_r) as confirmed by standards
231 were as follows: α-T $t_r = 5.5 \pm 0.1$ min, α-T3 $t_r = 7.0 \pm 0.1$ min, β-T $t_r = 9.8 \pm 0.2$ min, γ-T t_r
232 $= 11.0 \pm 0.2$ min, β-T3 $t_r = 13.0 \pm 0.2$ min γ -T3 $t_r = 14.7 \pm 0.3$ min, δ-T $t_r = 17.5 \pm 0.2$ min
233 and δ-T3 $t_r = 21.6 \pm 0.2$ min. This order and retention times of all peaks were as expected for
234 barley (Panfili et al., 2003). Amounts of δ-T3 and δ-T were considered to be negligible in all
235 barley genotypes since their minor peaks were not measurable.
236 The main tocols of the 25 genotypes detected, in descending order, were α-T3, α-T,
237 (γ-T3 and β-T3), (γ-T and β-T), δ-T3 and δ-T (Table 1). α-T3 was the main homologue,
238 accounting for approximately 58% of total tocols and 73% of total tocotrienol. All genotypes
239 also had significant amounts of α-T (~16% of total tocols), followed by γ-T3 (~12%) and β-

240 T3 (~10%). Smaller amounts of γ-T and β-T than other isomers were found in all samples.
241 The percentage of total tocotrienol (~80% of total tocols) was much higher than that of total
242 tocopherol (~19%).

243 The content of the six individual vitamin E isomers differed significantly between
244 genotypes with the 25 genotypes being easily categorised into three content groups: low,
245 medium and high. Jet, the ICARDA lines, Macumba, Sumire mochi and Tadmor had the
246 lowest content for the two main isomers, α-T and α-T3. Jet contained the lowest level of all
247 isomers except β-T. The high tocol content group comprised Harrington, ND24260-1,
248 Commander and Adagio. Harrington was richest in α-T3 and γ-T3 but also had relatively
249 high content of the other isomers. The remaining 12 genotypes had isomer contents that were
250 intermediate between the low and high groups.

251 There was a considerable range of vitamin E content between genotypes (Fig. 1a).
252 The genotype with the highest vitamin E content was Harrington (white, covered) with 31.47
253 μg/g DW, which is more than three times that of the lowest, Jet (hulless, black) with 8.53
254 μg/g DW. The hulless and coloured genotypes comprising Jet, Macumba, Finniss, Sumire
255 mochi, the ICARDA group and Tadmor were generally observed to have lower vitamin E
256 content than covered and white genotypes. When comparing between food, malt and feed
257 groups; the malting genotypes were richest in vitamin E content (~26 – 32 μg/g DW). In the
258 food group, Adagio was the best source of vitamin E with ~27 μg/g DW.

259 The average antioxidant capacity in the barley genotypes was ~109 mg AEAC/100 g
260 FW and the capacity ranged widely, from ~57 mg AEAC/100 g FW in ICARDA39 to ~158
261 mg AEAC/100 g FW in WI2585 (Fig. 1b). WI2585 and Harrington had the highest
262 antioxidant capacity and were also high in vitamin E, whereas the ICARDA lines were low in
263 both. As with vitamin E content, all coloured genotypes were lower in antioxidant capacity
264 than white genotypes (~57 – 104 mg AEAC/100 g FW compared with ~106 – 158 mg

265 AEAC/100 g FW). The malting genotypes tended to represent the best sources of
266 antioxidants (124.3±5.0 mg AEAC/100 g FW), followed by food (108.0±5.1 mg AEAC/100
267 g FW) and then feed genotypes (84.6±9.2 mg AEAC/100 g FW).

268 Along with the total antioxidant capacity analysis, the antioxidant capacity of vitamin
269 E was also examined (Fig. 1c) and found to be much lower than total antioxidant capacity
270 (~0.06 – 7 mg AEAC/100 g FW compared with ~57 – 158 mg AEAC/100 g FW), indicating
271 that vitamin E contributes a low proportion of the total antioxidant capacity in barley. In
272 addition, vitamin E and total antioxidant capacity were not significantly correlated ($n = 25$, r
273 $= 0.46$).

274

275 *3.2. Effect of storage on vitamin E and antioxidant capacity*

276 As expected, all six major isomers (α-T, α-T3, β-T, β-T3, γ-T and γ-T3) were detected
277 in barley genotypes after 4 months storage (Fig. 2), but the amounts of δ-T and δ-T3 were
278 negligible (data not shown). The ranking order for the concentration of isomers was similar to
279 that of non-stored samples, which was $\alpha > \gamma > \beta$. No significant storage effect was observed
280 for α-T in Macumba (Fig. 2a); α-T3 in ICARDA 19, Vlamingh, Tadmor, Commander or
281 Flagship (Fig. 2b); β-T in ICARDA 19, Sumire mochi, Macumba, Commander, Harrington,
282 Er/Apm or WI2585 (Fig. 2c); and γ-T in Sumire mochi (Fig. 2e). In this study, the change of
283 α-T among genotypes ranged from ~7 to 34%, whereas that of α-T3 was ~0.7 to 25% (Fig.
284 2a, 2b). As a results of these changes, overall vitamin E content changed by between 6 and
285 30% after 4 months of storages (Fig. 3).

286 The antioxidant capacity for most genotypes appeared lower in stored grains than
287 grains at harvest (Fig. 4). The reductions in antioxidant capacity in grains after storage were
288 lowest in Finniss (~6%) and highest in ND24260-1 (~16%). However, a significant increase
289 in antioxidant capacity was observed in the coloured genotypes ICARDA 26 and ICARDA

290 39. No difference was observed for ICARDA 19, ICARDA 35, Sumire mochi or Tadmor
291 (coloured genotypes) or Macumba, Flagship and Adagio (white genotypes).
292 PCA plots derived from the ATR-MIR analysis revealed that grains at harvest can be
293 differentiated from grain samples stored for 4 months (Fig. 5) using the first two principal
294 components, PC1 and PC2. The observed discrimination into different clusters based on
295 storage can be explained by the main MIR regions for each of the principal components
296 (Supplementary Information, Table S2). For the purpose of this paper only the lipid region
297 between frequencies 2900 cm^{-1} to 2800 cm^{-1} as well as 1750 cm^{-1} to 1700 cm^{-1} was analysed
298 and explain more than 90% of the variance in the PCA score plot.
299

300 **4. Discussion**

301 While a limited number of studies have investigated both antioxidant capacity and
302 vitamin E content in barley, no previous study has assessed these parameters across a number
303 of different genotypes. Our findings demonstrate that both vitamin E content and antioxidant
304 capacity are variable and genotype-dependent. Furthermore, declines in antioxidant capacity
305 and increases in vitamin E content were observed in most genotypes after 4 months of storage
306 at an industry relevant temperature.
307 The mean content of tocols for all barley genotypes was 72.9 µg/g DW, which is in
308 good agreement with those previously found by Peterson and Qureshi (1993) (58 µg/g DW),
309 Cavallero et al., (2004) (54.5 µg/g DW) and Panfili et al., (2008) (69.1 µg/g DW). The
310 average vitamin E content (24.2 µg/g DW) was also in good agreement with Panfili et al.,
311 (2008) (21.9 µg/g DW). However, the range of total tocol amount varied from 20.29 µg/g
312 DW for Jet (hulless/black/food type) to 102.43 µg/g DW for Harrington
313 (covered/white/malting type). This was much wider than that reported previously [42-80 µg/g
314 DW (Peterson & Qureshi, 1993); 51.0-61.4 µg/g DW (Cavallero et al., 2004) and 50.3-88.6

315 μg/g DW (Panfili et al., 2008)]. However, these previous studies included a much more
316 limited range of genotypes, and did not always include different classes. Therefore, our
317 results provide evidence that tocol content in barely is highly genotype-dependent. The
318 contribution of individual isomers to tocol content was similar to that found in other studies
319 (Ehrenbergerova, Belcrediova, Pryma, Vaculova, & Newman, 2006; Panfili et al., 2008). α-
320 T3 and α-T were most dominant in all barley genotypes, followed by β- and γ-tocols. The δ-
321 tocols, undetected isomers in the present study, were also not detected in other studies, or
322 made up less than 2 μg/g DW of content (Ehrenbergerova, Belcrediova, Pryma, et al., 2006;
323 Panfili et al., 2008). In the vitamin E pathway, α-tocols are derived from γ-tocols while β-
324 tocols are derived from δ-tocols (Hunter & Cahoon, 2007) which may explain the higher
325 content of α-tocols (compared to γ-tocols) and β-tocols (compared to δ-tocols).

326 Similar to tocol content, antioxidant capacity varied between the genotypes. Within
327 food genotypes, even though antioxidant capacity has been reported to be concentrated in the
328 husk (Peterson, 1994), the hulless genotypes used in this study, Macumba and Finniss, have a
329 relatively high antioxidant capacity (120 and 122 mg AEAC/100 g FW, respectively)
330 compared with the lowest antioxidant capacity genotype, Jet (57.22 mg AEAC/100 g FW)
331 and the highest antioxidant capacity genotype WI2585 (158.10 mg AEAC/100 g FW).
332 Macumba and Finniss, therefore, have potential for use in processing as they do not need to
333 be pearled.

334 Regardless of genotype, vitamin E only contributed a low proportion of the total
335 antioxidant capacity in barley grain. There was no correlation between antioxidant capacity
336 and the contents of five of the individual vitamin E isomers, however a significant correlation
337 was observed between antioxidant capacity and α-T3 content ($n=25$, $r=0.7$, $p<0.05$). For
338 example, Harrington had high antioxidant capacity and α-T3 content while ICARDA 39 had
339 low antioxidant capacity and α-T3 content. Although α-T has historically been reported as the

340 most efficient antioxidant (Mclaughlin & Weihrauch, 1979), α-T3 has recently been shown to
341 be at least three-fold more efficient as a scavenger of peroxyl radicals than α-T (Packer,
342 1995). α-T3 was the main vitamin E isomer in barley grain, regardless of genotype, and the
343 correlation with antioxidant capacity supports this observation.

344 All the coloured genotypes used in this study (the ICARDA lines, Jet, Sumire mochi
345 and Tadmor) showed lower levels of vitamin E than the white genotypes. These coloured
346 barley genotypes were also poor in antioxidant capacity (ICARDA lines > Jet > Sumire
347 mochi > Tadmor). Recent studies indicate that antioxidant capacity is high in coloured cereal
348 grains due to the contribution of pigment compounds such as proanthocyanidin and
349 anthocyanins, with the main forms being cyanidin-3-glucose and delphinidin-3-glucose
350 (Abdel-Aal, Young, & Rabalski, 2006). However, all eight coloured genotypes in this study
351 have lower antioxidant capacity than white genotypes, suggesting that non-pigmented
352 compounds may be more likely to contribute to antioxidant capacity in barley. Indeed, barley
353 has been previously reported to contain flavonol, phenolic acids and flavan-3-ols (Goupy et
354 al., 1999). Furthermore, antioxidant enzymes and apolar compounds combining tocols and
355 carotenoids (especially lutein and zeaxanthin) also appear to contribute to antioxidant
356 capacity in barley (Goupy et al., 1999). Given that vitamin E was probably not the main
357 contributor to antioxidant capacity in barley grain, the contribution of these other compounds
358 in the genotypes used in this study should be investigated further.

359 Vitamin E has recently been shown to be unstable in the presence of light, water,
360 oxygen and heat, particularly during extended periods of exposure (Wang et al., 1993). In the
361 current study, the changes after storage were different among genotypes, as well as among
362 their isomers, similar to the findings of Park, Kim, Park and Lee (2004) and Peterson (1995).
363 For example, the change in T was lower than that of T3. This can likely be attributed to the
364 difference in their molecular structures; T3 may be more susceptible to oxidation because of

365 its unsaturated side chains, whereas T has saturated side chains (Liu & Moreau, 2008).
366 Macumba and Commander were more stable than the other tested genotypes. Stability of
367 tocol isomer composition in barley has also been observed after storage for 11 months in a
368 conventional silo (Hakkarainen, Tyopponen, & Bengtsson, 1983), at ambient temperature for
369 8 weeks and 90°C for 48 hours (Tyopponen & Hakkarainen, 1985). The γ-T in Macumba
370 declined after storage, even though the other isomers remained unchanged. Given that γ-T is
371 the precursor to α-T in the vitamin E pathway, its decline suggests that the stability of α-T
372 was due to increased conversion of γ-T to α-T. All other genotypes showed increases in all
373 individual isomers after storage.

374 The changes in vitamin E content after storage differed between the genotypes, and
375 these differences are likely to be explained by the different initial content of vitamin E in the
376 respective genotypes before storage. Furthermore, each genotype appeared to have a different
377 fatty acid and lipid profile, indicating different ratios between unsaturated and saturated fatty
378 acids (data not shown). Indeed, the discrimination between samples before and after storage
379 observed in the PCA plots derived from the ATR-MIR analysis (Fig. 5) appears to be due to
380 differences in the frequencies associated with methyl groups, particularly in lipids.
381 Biochemical reactions changing the frequencies of methyl groups in lipids and the tocols
382 possibly associated with them may therefore occur during storage. This may lead to
383 differences in the extractability of lipids (Pomeranz & Chung, 1977) and therefore vitamin E.
384 In stored grain, reactions such as esterification may also prevent loss of vitamin E due to
385 reduction of vitamin E oxidation (Church & Pond, 1977).

386 Our findings are in good agreement with Liu and Moreau (2008) who found that
387 tocopherol contents increased in barley stored at 35°C as intact whole grains for 3 weeks or
388 25°C for 6 months. Wang et al., (1993) reported increasing δ-T3, while all T isomers
389 remained stable in barley after storage at 27°C. In contrast, some studies observed a decrease

390 in tocol isomers during storage. Tyopponen and Hakkarainen (1985) found that the tocol
391 content of barley flour which was stored and exposed to light at 25°C, decreased by 5% every
392 week over an 8 week period. At the same temperature, however, the degradation was less in
393 intact barley, with only 1% loss of tocols each month during 11 months of storage
394 (Hakkarainen et al., 1983). This discrepancy may be explained by differences in temperature,
395 light and moisture during storage. Heat may be an important factor in grain preservation
396 (Metz, 2006), and 10°C is considered to be an ideal storage temperature (Viljoen, 2001).
397 According to Ball (2006), T and T3 may be destroyed fairly rapidly by sunlight and artificial
398 UV light. The higher the moisture content, the shorter the shelf life of barley grains, for
399 example, barley grains with 11.5-12.5% moisture content stored for 3 months but barley
400 grains with 10.5-11.5% stored for 6 months (Metz, 2006). Furthermore, compared with
401 barley flour, tocols in intact barley grain degraded at a slower rate (Hakkarainen et al., 1983).
402 Storage time may also influence tocols. Interestingly, the content of α-T and α-T3 increased
403 during the first 2 to 3 months of storage but declined after 11 months storage in silos, from 92
404 to 20 μg/g DW (at 28% moisture content) or 80 to 15 μg/g DW (at 23% moisture content)
405 (Hakkarainen et al., 1983).

406 Our study showed that most of the barley genotypes, with the exception of coloured
407 genotypes, lost their antioxidant capacity after storage. This loss of antioxidant capacity after
408 storage has also been reported previously in wheat bran stored at 25°C (38% lost) or 60°C
409 (47% lost) after 9 days (Cheng, Su, Moore, Zhou, Luther, Yin et al., 2006) while the
410 enzymatic activities of dehydroascorbate reductase, glutathione reductase, glutathione
411 peroxidase and catalase decreased when grains were stored at 10°C (Spano, Bottega, Lorenzi,
412 & Grilli, 2011). The increase in antioxidant capacity observed in coloured barely genotypes
413 after storage in the present study is in agreement with the findings of Htwe et al., (2010) in
414 black and red rice during storage at 20, 30 and 40°C for up to four months. This result also

415 indicated that β-carotene in black rice increased whereas total anthocyanin remained stable
416 and both free soluble conjugated and insoluble bound forms of polyphenols decreased
417 significantly. Therefore, the relationship between increase in β-carotene content and higher
418 antioxidant capacity in coloured barley genotypes after storage needs further study.

419 As free radicals have been widely indicated as the major cause of seed deterioration
420 (Lehner, Mamadou, Poels, Come, Bailly, & Corbineau, 2008), the genotypes with higher
421 antioxidant capacity may be more likely to be preserved during storage and processing. Other
422 authors have previously shown that processed oat products were more stable when the oat
423 genotype contained higher antioxidant levels (Peterson, 2001). Antioxidants have also been
424 shown to act as a preservative when added to various foodstuffs. This is the case not only for
425 pure antioxidants but also for extracted antioxidants; oat hull extract, for example, can inhibit
426 fungal and bacterial growth (Peterson, 2001). Therefore, grains with inherent antioxidants
427 may have more potential to protect themselves in storage. This is in accordance with the
428 results of a previous study of stored wheat, which showed a negative correlation between the
429 efficiency of the antioxidant enzymatic machinery and the age of grain (Lehner et al., 2008;
430 Spano et al., 2011). Possible future research could be aimed at investigating the relationship
431 between high antioxidant genotypes and their shelf life during storage.

432

433 5. Conclusions

434 The genotypic differences identified here will allow genotypes with high antioxidant
435 capacity and/or vitamin E content to be chosen for breeding purposes. The genotypes that
436 maintain antioxidant capacity and/or vitamin E content after storage have the best potential
437 for functional food products. We are now determining the genetic basis of differences in
438 antioxidant capacity and/or vitamin E content by evaluating mapping populations derived
439 from parents with low or high antioxidant capacity or vitamin E content.

440

441

442 **Supplementary information**

443

444 Supplementary Fig. S1: Representative chromatogram of tocols in barley in this study.

445

446 Supplementary Table S1: Barley genotypes and their characteristics.

447

448 Supplementary Table S2: The loadings in the first two principal components at wavenumbers

449 associated with certain molecules.

450

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455

456 **Abbreviations**

457 DPPH, 2,2-diphenyl-1-picrylhydrazyl; MIR, Middle InfraRed; NIR, Near InfraRed;

458 ATR, Attenuated total reflectance; AEAC, Ascorbic Acid Equivalent Antioxidant Capacity;

459

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566

Table 1. **Mean content of tocopherols and tocotrienols in 25 barley genotypes at harvest** (for n=6 biological replicates except n=9 for Jet and ICARDA 16 and n= 3 for Buloke).

	Content (µg/g DW)								
Genotype	α-T	β-T	γ-T	Total T	α-T3	β-T3	γ-T3	Total T3	Tocols T+T3
Jet	4.62^{a}	0.34^{efgh}	0.29^{a}	5.25	12.18^{a}	1.55^{a}	1.31^{a}	15.04	20.29
Sumire mochi	8.60^{b}	0.40^{efghi}	1.04^{b}	10.04	38.36^{de}	3.45^{c}	8.24^{fg}	50.05	60.09
Macumba	8.93^{b}	0.40^{efghi}	0.40^{a}	9.73	35.96^{d}	3.51^{c}	8.10^{efg}	47.57	57.30
Finniss	9.55^{c}	0.42^{fghij}	0.32^{a}	10.29	43.59^{gh}	7.92^{lm}	7.47^{e}	58.98	69.27
ICARDA 16	13.15^{k}	0.25^{abcd}	2.60^{jk}	16.00	44.01^{h}	7.31^{jk}	4.50^{c}	55.82	71.82
ICARDA 19	14.47^{l}	0.26^{bcde}	5.70^{q}	20.43	32.34^{c}	2.84^{b}	4.33^{c}	39.51	59.94
ICARDA 26	12.59^{ij}	0.13^{a}	2.38^{hi}	15.10	27.38^{b}	4.00^{d}	4.37^{c}	35.75	50.85
ICARDA 35	11.00^{de}	0.33^{cdefg}	1.63^{d}	12.96	33.42^{c}	4.14^{d}	4.13^{c}	41.69	54.65
ICARDA 39	12.17^{ghi}	0.20^{ab}	2.78^{k}	15.15	28.70^{b}	6.41^{gh}	2.73^{b}	37.84	52.99
Tadmor	10.03^{c}	0.44^{ghij}	1.90^{f}	12.37	40.71^{ef}	7.70^{kl}	6.49^{d}	54.90	67.27
Er/Apm	10.59^{d}	0.29^{bcde}	1.30^{c}	12.18	43.91^{h}	6.81^{hi}	11.37^{jk}	62.09	74.27
Vlamingh	11.83^{fg}	0.31^{bcdef}	2.19^{gh}	14.33	51.89^{ij}	6.18^{g}	8.48^{g}	66.55	80.88
Commander	12.39^{hij}	0.49^{ij}	2.06^{fg}	14.94	55.40^{k}	8.38^{n}	9.88^{h}	73.66	88.60
Buloke	12.06^{gh}	0.46^{hij}	5.03^{p}	17.55	44.56^{h}	10.06^{p}	13.36^{m}	67.98	85.53
Fleet	12.10^{ghi}	0.43^{fghij}	1.89^{ef}	14.42	45.84^{h}	10.50^{pq}	11.40^{jk}	67.74	82.16
Flagship	11.33^{ef}	0.29^{bcde}	2.08^{fg}	13.70	46.06^{h}	7.56^{kl}	8.04^{efg}	61.66	75.36
Sloop	10.79^{d}	0.40^{efghi}	1.13^{bc}	12.32	51.11^{i}	13.56^{r}	10.21^{i}	74.88	87.20
Chebec	12.80^{jk}	0.63^{k}	2.07^{fg}	15.50	45.93^{h}	17.77^{s}	12.52^{l}	76.22	91.72
Amaji nijo	13.29^{k}	0.48^{ij}	4.58^{n}	18.35	41.27^{fg}	4.60^{e}	14.81^{n}	60.68	79.03
Harrington	12.21^{ghi}	0.45^{ghij}	2.54^{ij}	15.20	60.43^{l}	10.74^{q}	16.06^{o}	87.23	102.43
WI2585	11.70^{fg}	0.66^{k}	3.74^{l}	16.10	44.35^{h}	7.01^{ij}	11.61^{k}	62.97	79.07
Dhow	12.00^{gh}	0.23^{abcd}	1.70^{de}	13.93	54.05^{jk}	9.48^{o}	10.89^{ij}	74.42	88.35
ND24260-1	12.58^{ij}	0.35^{efgh}	3.74^{l}	16.67	50.36^{i}	6.06^{g}	7.62^{ef}	64.04	80.71
Adagio	12.75^{jk}	0.54^{jk}	4.69^{no}	17.98	45.93^{h}	8.22^{mn}	11.99^{kl}	66.14	84.12
Alexis	12.45^{hij}	0.21^{abc}	4.23^{m}	16.89	51.08^{i}	5.07^{f}	10.45^{i}	66.60	83.49
Mean	11.44	0.38	2.48	14.30	42.56	7.23	8.81	58.60	72.90
LSD	0.52	0.12	0.19		2.51	0.45	0.68		

The same letter indicates no difference between genotypes for individual isomers (within column) as determined using the Least Significant Difference (LSD) ($P<0.05$). δ-T3 and δ-T were not detected.

580 Figure Captions

581

582 **Fig. 1 Vitamin E contents (a), antioxidant capacity (b) and antioxidant capacity of**
583 **vitamin E (c) in 25 barley genotypes at harvest, shown in ascending order.** The names
584 marked with * or with ** are feed or food genotypes, respectively. The others are malting
585 genotypes. Vitamin E is expressed in mg of α-tocopherol-equivalents (TE) and antioxidant
586 capacity is expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g
587 of fresh weight (FW) of grain. Bars represent the mean ± SE. For a, n=6 except n=9 for Jet
588 and ICARDA 16 and n=3 for Buloke. The Least Significant Difference (LSD) (P<0.05) =
589 1.03. For b, n=4 except n=7 for Finniss, Adagio, Sumire mochi, Macumba, Tadmor, Jet,
590 Commander, Chebec, Harington, Sloop, Er/Apm, Flagship and n=3 for Buloke. The LSD
591 (P<0.05) = 7.66. For c, n =3 for all genotypes. The LSD (P<0.05) = 0.61.

592

593 **Fig. 2 Effect of storage on the profile of tocopherol and tocotrienol isomers in different**
594 **barley genotypes.** α-T (a), α-T3 (b), β-T (c), β-T3 (d), γ-T (e), and γ-T (f). The genotype
595 names marked with * or with ** are feed or food genotypes, respectively. The others are
596 malting genotypes. □, content before storage, n=6 except n=9 for Jet and ICARDA 16, and
597 n=3 for Buloke; ■, content after storage, n=3 for all stored genotypes. The bars represent
598 means (±SE). The Least Significant Difference ($LSD_{sample.time}$) (P<0.05) for α-T=0.65;
599 $LSD_{sample.time}$ for α-T3=3.11; $LSD_{sample.time}$ for β-T=0.18; $LSD_{sample.time}$ for β-T3=0.57;
600 $LSD_{sample.time}$ for γ -T=0.39; $LSD_{sample.time}$ for γ-T3=0.95.

601

602 **Fig. 3 The vitamin E content of grains from different barley genotypes before and after**
603 **storage.** The names marked with * or with ** are feed or food genotypes, respectively. The
604 others are malting genotypes. □, content before storage, n=6 except n=9 for Jet and ICARDA

605 16, and n= 3 for Buloke; ■, content after storage, n=3 for all stored genotypes. Bars present
606 the mean ± SE. The Least Significant Difference samples by time ($LSD_{sample.time}$)
607 (P<0.05)=1.40.

608

609 **Fig. 4 The percentage change of antioxidant capacity of grains from different barley**
610 **genotypes after storage**. The names marked with * or with ** are feed or food genotypes,
611 respectively. The others are malting genotypes. ■, colour genotypes; □, non-colour
612 genotypes. Bars not within dotted lines represent genotypes with significantly different
613 percentage change of antioxidant capacity after storage.

614

615 **Fig. 5 Score plot of the two principal components of barley flour samples before and**
616 **after storage analysed using attenuated total reflectance and mid infrared spectroscopy.**
617 PC1, principal component 1; PC2, principal component 2.

618

619