

1 **Influence of drought stress on Alfalfa yields and nutritional**
2 **composition**

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4 Yinghao Liu¹, Qian Wu², Gentu Ge³, Guodong Han⁴, Yushan Jia^{*1}

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6 Yinghao Liu¹, E-mail Address:s16414332395@126.com

7 Qian Wu², E-mail Address:532759133@qq.com

8 Gentu Ge³, E-mail Address:gegentu@163.com

9 Guodong Han⁴, E-mail Address:hanguodong@163.com

10 Yushan Jia^{*1}, E-mail Address:610480018@qq.com

11 **ABSTRACT**

12 It is predicted that climate change may increase the risk of local droughts, with severe
13 consequences for agricultural practices. Here we report the influence of drought on
14 alfalfa yields and its nutritional composition, based on artificially induced drought
15 conditions during two field experiments. Two types of alfalfa cultivars were
16 compared, Gold Queen and Suntory. The severity and timing of a drought period was
17 varied, and the crop was harvested either early during flowering stage, or late at full
18 bloom. The obtained dry mass yields of Gold Queen were higher than Suntory, and
19 the first was also more resistant to drought. Early harvest resulted in higher yields.
20 Decreases in yields due to water shortage were observed with both cultivars, and the
21 fraction of crude protein (CP) decreased as a result of drought stress; this fraction was
22 higher in Gold Queen than in Suntory and higher in early harvest compared to late
23 harvest. Severe drought late in spring had the highest effect on CP content. The

24 fraction of fibre, split up into neutral detergent fibre (NDF) and acid detergent fibre
25 (ADF) increased as a result of drought and was lower in early harvested plants
26 compared to late harvest. Suntory alfalfa produced higher fibre fractions than Gold
27 Queen. The fraction of water-soluble carbohydrates (WSC) was least affected by
28 drought. It was consistently higher in Gold Queen compared to Suntory alfalfa, and
29 late harvest resulted in higher WSC content. In combination, these results suggest that
30 the nutritive value of alfalfa will likely decrease after a period of drought. These
31 effects can be partly overcome by choosing the Gold Queen cultivar over Suntory, by
32 targeted irrigation, in particular in late spring, and by harvesting at an earlier time.

33

34 **Key words:** alfalfa (*Medicago sativa* L.), drought stress, water regimes, nutritive
35 value.

36

37 INTRODUCTION

38 Grassland remains an important feed source for ruminant nutrition, with its high
39 productivity and good fodder quality¹, but alfalfa is often a necessary feed additive or
40 alternative, especially suitable for feed production under nitrogen-limiting conditions,
41 due to the plant's ability to fix atmospheric N². With the increase of energy costs,
42 fertiliser (as an artificial source of soil N) has become more expensive, a trend that is
43 expected to continue in the future, which will likely further increase the need of
44 legume production, including alfalfa^{3,4}. Agricultural forage production depends on an
45 adequate water supply⁵, a dependence that can become problematic in semi-arid
46 climates, especially where local effects due to climate change increase the probability

47 of summer droughts⁶⁻⁸. Insufficient water supply can have strong effects on the
48 production of forage legumes⁹, resulting in a decrease in yield depending on the
49 severity and duration of drought stress^{10,11}.

50 It is well known that forage legumes differ in drought stress sensitivity¹². White
51 clover is one of the most important legumes in agricultural production, but it is also
52 relatively drought sensitive¹³. There is limited and inconsistent knowledge available
53 about the influence of drought stress on the nutritive value of alfalfa. Under such
54 conditions, concentrations of acid detergent fibre (ADF) and neutral detergent fibre
55 (NDF) were found to be reduced in a range of forage legumes, while inconsistent
56 changes were reported in crude protein (CP) concentrations¹⁴. A different study
57 described an increase in ADF with a minor effect only for CP and NDF
58 concentrations in red clover and alfalfa¹⁵. Another study reported an increase in
59 water-soluble carbohydrates (WSC) under water shortage in two cultivars of soybean
60 but it did not include alfalfa¹⁶. For clover species, only a small drought-induced effect
61 on WSC was observed¹⁷. Alfalfa is possibly less sensitive to drought than white
62 clover, but more research is needed to predict the influence of drought stress on the
63 nutritive value of alfalfa, which was therefore one of the aims of this study.

64 Alfalfa was used here to examine the effects of drought stress on the
65 concentrations of CP, WSC and the fibre components NDF and ADF, which were
66 chosen as indicators for nutritive value. The CP concentration is an essential
67 component for ruminant nutrition, and is typically high in alfalfa due to effective N
68 fixation; WSC have a positive influence on fodder intake and are important for an
69 efficient utilisation of dietary N; NDF content provides an estimate of the cellulose,

70 hemicellulose and lignin content and is inversely related to voluntary fodder intake;
71 finally ADF includes lignin and cellulose and is negative correlated with cell wall
72 digestibility¹⁸⁻²⁰¹⁶⁻¹⁸. In the present study the following questions were addressed: (1)
73 do different alfalfa species differ in their response to drought stress; and (2) what is
74 the effect of timing, duration, and intensity of water restriction on the nutritional
75 parameters of alfalfa.

76

77 **Results**

78 In two field experiments, conducted in two consecutive years, two cultivars of alfalfa
79 were subjected to various levels of drought: Gold Queen alfalfa and Suntory alfalfa.
80 The plants were harvested at two developmental stages. In Experiment I, conducted in
81 2013, two drought levels were simulated during spring (moderate drought in condition
82 C1, and severe drought in C2) and two drought levels were applied in summer
83 (moderate, C3, and severe drought); optimally watered plots (CO) served as control.
84 In Experiment II, conducted a year later, severe drought was implemented either in
85 early spring (C5) or in late spring (C6), while optimal watering (CO) was also
86 compared to natural rainfall without further irrigation (C7). In both experiments, for
87 all conditions, half of the plots were harvested early (samples marked 'E'), during the
88 initial blooming phase, and the other half late (samples marked 'L') at full blooming
89 stage. The conditions tested are summarized in **Table 1**. All plots within one
90 experiment were harvested at the same time, and the harvested alfalfa was chemically
91 analyzed for a number of nutritional variables.

92 **Figure 1** shows the relative available water in the soil during treatments C1-C4

93 (experiment I, 2013) and C5-C7 (experiment II, 2014). A plot of local precipitation
94 and temperature during the experiments are available as **Supplementary Figure S1**.
95 In 2013, spring was warm and dry while summer temperatures were moderate. Yearly
96 total rainfall amounted to 224 mm with an average temperature of 16.9°C (Fig. S1).
97 The year 2014 started with low temperatures, followed by a cool spring, with total
98 rainfall (327 mm) and average air temperature (15.7°C) lower than in 2013. The
99 beginning of 2014 was relatively cool, which delayed vegetation development, while
100 May was unusually wet with over 100 mm rainfall (Fig. S1).
101 The alfalfa early and late yields in Kg/ha, after removal of weeds and after drying (dry
102 mass, DM), are shown in Table 1.

103 **Crude protein concentration**

104 Crude protein concentrations from the harvested alfalfa were determined and
105 expressed as percentage of dry mass (% DM) (**Table 2**). Whereas moderate drought
106 applied during the spring of 2013 (C1) had no significant effect on CP concentration,
107 severe drought (C2) resulted in significantly lower CP values when compared to
108 optimally watered controls (CO), for both alfalfa types, and for both harvest times
109 (Table 2). ANOVA statistical analysis indicated there was a significant difference
110 ($P < 0.001$) for CP content between the compared cultivars, with Gold Queen alfalfa
111 producing significantly higher CP contents under all conditions tested. Early harvest
112 produced significantly higher yields than late harvests, for both cultivars. The overall
113 range of CP content of alfalfa grown with drought stress during spring varied between
114 15.61 and 17.61% DM. When drought stress was applied during the summer of 2013,
115 CP content varied between 14.68 and 18.73% (Table 2), a range that was not

116 significantly different to the range obtained with spring drought stress. The response
117 of the two alfalfa cultivars to drought stress applied in summer was again significantly
118 different, and CP content was lower when drought stress was severe (C4) compared to
119 moderate stress (C3). As before, CP content was higher in Gold Queen alfalfa than in
120 Suntory alfalfa, for all conditions tested. The ANOVA analysis further indicated that
121 as a result of summer drought stress, Gold Queen suffered significantly more than
122 Suntory alfalfa, resulting in a significance for DSxCV (Table 2).

123 In 2014, the timing of drought stress during spring was varied. A dry period early in
124 spring (C5) reduced CP content of alfalfa compared to the optimally watered control
125 (CO), with the exception of the Gold Queen cultivar harvested during the initial
126 flowering phase (C5E), which actually contained a higher ratio of CP compared to the
127 control (Table 2). A period of drought in late spring (C6) affected all plots, except for
128 Gold Queen alfalfa harvested early (C6L). Natural rainfall without irrigation (C7)
129 significantly reduced the CP content (Table 2). Statistical ANOVA analysis indicated
130 that the combination of drought stress and harvest time produced significantly
131 different results (a late harvest after late-spring drought produced lowest yields).
132 Likewise, the combined factors of drought stress and cultivar, of harvest time and
133 cultivar, and the combination of all three parameters were significant. The highest CP
134 content was obtained with Gold Queen harvested early following a severe drought in
135 early spring. The lowest CP content was obtained with Suntory alfalfa harvested late
136 following a severe drought in late spring.

137 **Concentration of neutral and acid detergent fiber**

138 The concentration of neutral detergent fibre (NDF) was higher in Suntory than in

139 Gold Queen alfalfa, but the difference was not significant ($P=0.0908$). Early harvest
140 resulted in a significantly lower fraction of NDF than late harvest (**Table 3**).
141 Independently of the time of harvest, the content of NDF was increased by severe
142 drought stress in spring (C2) or summer (C4), though a moderate drought during
143 spring (C1) or summer (C3) had no effect on NDF content. During spring, both an
144 early and a late drought (C5 and C6) increased the NDF fraction equally (Table 3).
145 The concentration of fibre extracted with acid detergent (ADF) in part followed the
146 same trends as those of NDF. ADF was also higher in Suntory than in Gold Queen,
147 and this difference was now significant (**Table 4**). As was observed for NDF, late
148 harvest increased the fraction of ADF, and both an early and a late period of drought
149 during spring significantly increased the ADF fraction, as shown in Table 4. However,
150 the ADF fraction was reduced under severe drought in spring (C2) and after a
151 moderate drought in summer (C3), while a severe drought in summer (C4) increased
152 the ADF fraction (Table 4).

153 **Concentration of water-soluble carbohydrates**

154 The fraction of water-soluble carbohydrates (WSC) was significantly higher in Gold
155 Queen than in Suntory alfalfa under all tested conditions, and late harvest produced
156 higher fractions (**Table 5**). In Experiment I drought stress did not significantly affect
157 these fractions, but in Experiment II, lower WSC fractions were obtained following a
158 drought period in spring, with the exception of early harvested Suntory (Table 5).

159 **Hay yield reduction**

160 At the end of the experiment all plant material was cut 3 cm above the surface and
161 dried. Hay yields in Gold Queen alfalfa and Suntory alfalfa were reduced by all

162 water-restricted conditions (**Table 6**), with a reduction between 27% (rain fed, C7)
163 and 83% (Gold Queen alfalfa, draught in late spring, C6).

164

165 **Discussion**

166 The field experiments described here were conducted to assess the effect of water
167 restriction regimes on two alfalfa cultivars, by the use of fixed and mobile rain
168 shelters. The validity of such an approach to study microclimatic effects has been
169 convincingly demonstrated before²¹. We are aware that the shelters may have
170 increased the temperature above ground, especially during hot days in summer, which
171 may have affected plant development²², as it would add heat stress to the plants in
172 addition to drought stress. Experiments conducted in winter would not suffer from this
173 combined effect, possibly resulting in smaller changes, for instance like those reported
174 for grain yield studied in winter wheat²³. However, since reduced rainfall as a result of
175 local climate changes often coincides with higher than normal temperatures, we
176 believe our experimental conditions sufficed to investigate their combined effect.
177 Although temporarily increased day temperatures might have negatively affected the
178 plant growth and the yield of alfalfa, water limitation was most likely the main driver
179 of the observed changes. Irrespective of the water supply treatment, the analytical data
180 of the harvested alfalfa resulted in a predictive nutritive value comparable to those
181 found in the literature^{14,24}. With the values obtained, the harvested alfalfa would be
182 considered of a moderate to high quality feed^{25,26}. Under optimal water conditions, the
183 parameters for feed quality were better for Gold Queen alfalfa than for Suntory alfalfa,
184 with higher protein and lower fiber contents, though the higher water-soluble

185 carbohydrate fraction in Gold Queen could be considered less beneficial.

186 The fraction of crude protein was reduced as a result of drought, with a similar
187 decrease in both cultivars, but even so, CP content remained higher in Gold Queen
188 than in Suntory. The CP fraction generally depends on the amount of available N^{1,27}
189 and alfalfa is particularly effective in N-fixation. When the N fixation performance of
190 alfalfa was determined, it produced 10 to 30% higher fixation levels than other
191 legumes²⁸. Thus, the degree of nitrogen fixation determines the availability of N for
192 protein production, but it is not the only limiting factor for biomass production:
193 obviously, this is also determined by water availability. Experiments with soybean
194 identified N uptake as an important factor for biomass production under drought¹⁶,
195 and peanut plants decreased their N fixation under drought stress²⁹. We interpret the
196 decrease in CP fractions in drought-stressed alfalfa to be caused by a combined stress
197 response to water limitation in addition to a decrease in N fixation.

198 The content of neutral fiber increased under strong drought stress, a change that was
199 also observed for acid-extracted fiber under certain conditions, though mixed results
200 were obtained for the latter. Fiber concentration is influenced by many interacting
201 factors, such the phase of plant development, leaf-stem ratio, environmental
202 conditions (water, temperature, available light etc.), and availability of nutrients²⁴⁻²⁶.

203 The increase in NDF and ADF fractions under stress is not supported by findings in
204 the literature, where a delayed maturity under drought was reported, associated with
205 lower NDF and ADF concentrations^{14,25}. The major difference between the NDF and
206 the ADF fraction is that the former included hemicellulose (the other main
207 components are cellulose and lignin for both fractions), and the stronger and more

208 consistent increase of the NDF as a result of drought stress in alfalfa suggests that
209 production of hemicellulose is most affected by water restriction. However, results on
210 the effects of drought on hemicellulose concentrations are inconsistent in the literature,
211 as some authors have reported decreased hemicellulose concentration under drought,
212 while other reported an increase³⁰. We found that the ADF concentration was
213 consistently lower than that of NDF, a finding that has been reported for other
214 legumes and for most grasses as well²⁶. A lower fiber concentration is generally
215 considered beneficial, as it may lead to a higher herbage intake and to an increase in
216 digestibility of forage. An early harvest resulted in lower fiber content and this,
217 combined with a higher protein content, suggests that harvesting early in the season
218 may improve the quality of the alfalfa, particularly after drought.

219 The fraction of WSC was least affected by drought stress, showing only a minor
220 decrease as a result of drought stress in spring, although others have reported an
221 increase as a result of drought in other plant species^{16,31}. Gold Queen alfalfa contained
222 significantly higher fractions of WSC, which might explain why it was also generally
223 more capable to cope with drought stress. A high WSC concentration in plants would
224 result in a higher osmotic potential, which drives the uptake of soil water and is
225 therefore of importance to minimize drought stress effects³². This osmotic adjustment
226 is a physiological mechanism in response to drought¹⁶, but in our experiments the
227 WSC content changed marginally, only producing a significant decrease during spring
228 drought.

229 Without irrigation, yields were low and nutritional parameters poor, as demonstrated
230 with the samples grown under natural rainfall. When water supply is limited and

231 continuous irrigation may not be possible, the timing of irrigation needs to be
232 carefully considered. Our results indicate that the most beneficial effect can be
233 expected if irrigation prevents a severe drought in late spring.
234 Digestibility of fodder may decrease under strong drought stress due to a tendency to
235 lower WSC and higher fiber fractions, and combined with lower protein content this
236 would reduce the nutritive value. However, the decreased protein to fiber ratio in alfalfa
237 following a drought would result in a decrease of nitrogen secretion in the urine of
238 ruminants²⁰, which can be considered beneficial for the environment. The choice of
239 cultivar (Gold Queen) and an early harvest can minimize drought effects. Animal
240 experiments need to be performed to further assess the feed-to-weight conversion and
241 waste production of alfalfa grown under drought stress.

242

243 **Conclusions**

244 The production of alfalfa is a main agricultural activity in areas of China where
245 relatively sandy and infertile soils do not allow many other crops to be produced, but
246 in particular these areas are expected to suffer from increased periods of drought as a
247 result of climate change. It is therefore important to anticipate possible changes in the
248 nutritive value of alfalfa as a result of drought stress. We have demonstrated that only
249 severe drought stress has an impact on yield and composition of alfalfa. Strong
250 drought led to a decrease in hay yield, a decrease in CP content, and an increase in
251 fibre. These effects might in combination decrease the digestibility of the herbage.
252 However, as the ratio of CP to WSC decreased under drought, this could reduce the N
253 surplus in ruminates. We observed differences between the two tested alfalfa cultivars,

254 both in their performance under optimal water supply and in their response to drought
255 stress, with Gold Queen performing better than the Suntory cultivar. Finally, an early
256 harvest could minimize the effects of drought. The reported findings may assist
257 farmers in choosing the best cultivar, irrigation strategy and harvesting time, to
258 mitigate the effect of decreased precipitation that can be expected in the future.

259

260 **Material and methods**

261 **Field experiments.** A field study was conducted in 2012-2014 in a vegetation area of
262 Ar Horqin Banner near the Nei Monggol Autonomous Region, China (coordinates
263 37°43'N; 120°22'E), using a randomized complete blocks design with three variable
264 parameters tested with four replications. Two types of alfalfa were compared: Gold
265 Queen and Suntory alfalfa. Conditions resembling severe draught and moderate
266 draught were compared with optimal water supply whereby the timing of water
267 restriction was also varied. The plants were harvested early (samples designated 'E')
268 during the initial flowering phase, or late ('L') during full bloom. Details about the
269 applied water regimes are described in **Table 1**. Drought stress was imposed during
270 three periods with varying severity. The trials were divided into two experiments. In
271 Experiment I (2013, spring through summer, sowing date 23 July 2012, harvest date
272 14 September 2013), three restricted water supply regimes were compared to optimal
273 water supply: severe drought stress during spring, and moderate and severe drought
274 stress during summer. Moderate stress corresponded with 20-40% usable water
275 capacity of the soil and severe stress corresponded to only 10-15%. In Experiment II,
276 conducted a year later (sowing date 16 August 2013, harvest date 21 September 2014),

277 two water restriction conditions (15% available water), in early spring and in late
278 spring, were compared to rain fed and optimally watered plots.

279 Drought stress was implemented by restricting rain precipitation on individual plots,
280 using a 12m long, 6m wide, and 5m high foil cover (CASADO, Dou-ville, France).
281 This stationary shelter was covered by 200- μ m polythene foil, which was mounted
282 over the plot. In order to attain good ventilation and to minimize microclimatic effects
283 of the shelter, the front and the sides were left open.

284 **Alfalfa types.** In both experiments two alfalfa genotypes were examined; Gold Queen
285 alfalfa is a novel American cultivar, marketed as being salt-tolerant, and suitable for
286 high saline-alkali soil. Suntory alfalfa is a French cultivar, which has a high yield of
287 high quality and is known to be disease-resistant.

288 The seeds were kindly provided by the College of Agriculture and Animal Husbandry,
289 ChiFeng, China. The seeding density was 0.5 kg seed per 0.667 hm² and plot sizes
290 ranged from 6.6 to 8.2 m².

291 **Climate conditions.** Air temperature and precipitation were recorded at 2 m height
292 with a iMETOS weather station (Pessl Instruments, Weiz, Austria) located on the
293 experimental field. The agrometeorological advisory system from the China Weather
294 Service (CWS, 2014) was used to plan irrigation scheduling.

295 **Soil composition and water content.** The soil was characterized as Haplic Luvisol
296 with an available water capacity of 120 mm (0–90 cm), and a groundwater level 10 m
297 below surface. The soil was composed of 36% corn soil, 27% sand, 12% chernozem
298 and 5% of other components. The pH of the soil (in CaCl₂ suspension) measured in
299 summer 2013 was 7.3. The soil moisture was recorded during the experiments using a

300 portable soil moisture probe Diviner 2000 (Santé Technologies, Stepney, Australia).
301 Plastic tubes with a diameter of 5 cm were installed to a depth of up to 150 cm. Soil
302 moisture readings were taken at 10 cm intervals from 5 to 125 cm three times per
303 week from the beginning of vegetation to harvest. The soil water content was also
304 determined gravimetrically on several occasions in order to obtain a site-specific
305 calibration ($R^2 = 0.64$). The soil moisture data over time are presented as ml/100 g
306 soil (%).

307

308 **Sampling and measurements.** For each experiment, plots were harvested by hand.
309 For harvests, over an area of 0.09 m² (in Experiment 1, 0.18 m² in Experiment 2) per
310 plot the plants were cut at a height of 3-4 cm above the soil surface and cuttings were
311 separated from weeds immediately after harvest. Dry weight of alfalfa was
312 determined after drying at 60°C for 72 h in a drying oven (ULM 800, Member GmbH,
313 Schwa Bach, Germany).

314 For analysis of CP, NDF, ADF and WSC, dried samples were ground to 1 mm and
315 these were analysed by near-infrared reflectance spectroscopy (NIRS). All findings
316 are reported as % dry mass (%DM). The spectra were analysed using the large dataset
317 of calibration samples from different kinds of grasslands by the Institute VDLUFA
318 Qualitätssicherung NIRS GmbH, Kassel, Germany.

319 **Statistical analyses.** Analyses of variance were carried out with the GLIMMIX
320 procedure of SAS 9.3 (SAS Institute, Cary, NC, USA). We did a three factorial
321 analysis of variance (ANOVA) for CP, NDF, ADF and WSC concentrations of two
322 species in initial flowering phase and full-bloom phase of the harvest following each

323 stress period (Payne, 2002). Experiment I and II as well as individual years were
324 analyzed separately because of different water regimes among the years. Correlations
325 were calculated with the CORR procedure of SAS. Graphs were created with
326 SigmaPlot 12 (Systat Software Inc., Chicago, IL, USA). The three factors were
327 legume species (LS), flowering phase (FS) and drought stress (DS). Relationships
328 between selected variables were examined with a linear regression model.

329

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434

435 **Author Contributions**

436 Liu YH and Jia YS conceived the study. Ge GT and Liu YH designed the experiments.
437 Fan WQ, Wang ZJ, Han GD and Liu TY performed the fieldwork. Wang W and Liu

438 XB supervised the fieldwork. Yin Q performed the quantitative data analysis. Liu YH
439 wrote the manuscript. All authors reviewed and approved the manuscript.

440

441 **Additional Information**

442 Competing financial interests: The authors declare no competing financial interests.

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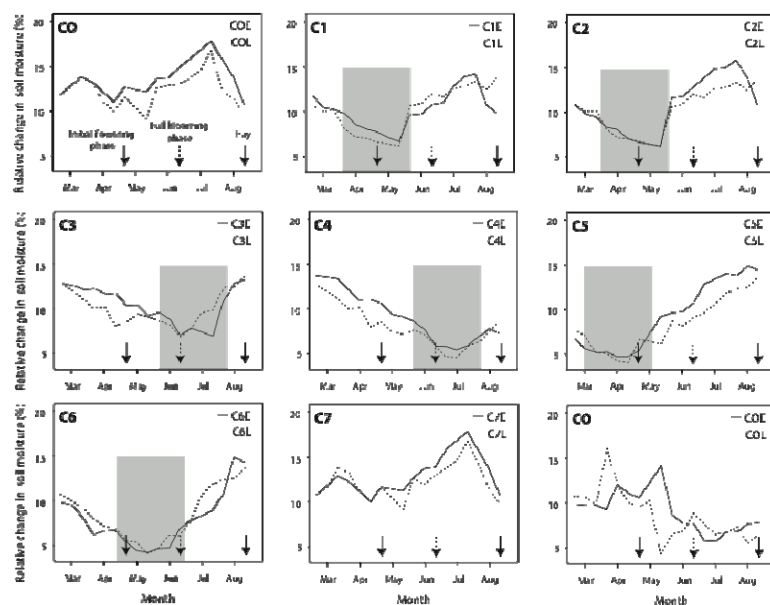


Figure 1. Soil moisture levels (mg water/100 mg soil) over time for optimally watered controls (CO) and experimental conditions C1-C4 (Experiment 1) and C5-C7 (Experiment 2). In each plot, two average moisture levels of four individual plots are shown, for those harvested early (solid lines) and late (dotted lines). The periods of artificially induced drought are indicated in grey. The three arrows indicate the time of early harvest, late harvest and final harvest.

Supplementary Figure S1

Fig. S1. Air temperature and rainfall in the two years during which the experiments were conducted.

