Plastic Response of *Medicago sativa* L. root system traits and cold resistance to simulated rainfall events

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Climate change (rainfall events and global warming) affects the survival of alfalfa (*Medicago sativa* L.) in winter. Appropriate water management can quickly reduce the mortality of alfalfa during winter. To determine how changes in water affect the cold resistance of alfalfa, we explored the root system traits under different rainfall events and the effects on cold resistance in three alfalfa cultivars. These were exposed to three simulated rainfall events (SRE) × two phases in a randomized complete block design with six replications. The three cultivars were WL168, WL353 and WL440, and the three SRE were irrigation once every second day (D_2), every four days (D_4) and every eight days (D_8). There were two phases: before cold acclimation and after cold acclimation. Our results demonstrated that a period of exposure to low temperature was required for alfalfa to achieve maximum cold resistance. The root system tended toward herringbone branching under D_8, compared with D_2 and D_4, and demonstrated greater root biomass, crown diameter, root volume, average link length and topological index. Nevertheless, D_8 had less lateral root length, root surface area, specific root length, root forks and fractal dimensions. Greater root biomass and topological index were beneficial to cold resistance in alfalfa, while more lateral roots and root forks inhibited its ability to survive winter. Alfalfa roots had higher proline, soluble sugar and starch content in D_8 than in D_2 and D_4. In contrast, there was lower malondialdehyde in D_8, indicating that alfalfa had better cold resistance following a longer irrigation interval before winter. After examining root biomass, root system traits and physiological indexes we concluded that WL168 exhibited stronger cold resistance. Our results contribute to greater understanding of root and cold stress and, consequently, provide references for selection of cultivars and field water management to improve cold resistance of alfalfa in the context of changes in rainfall patterns.

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13 **Abstract**

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15 *sativa* L.) in winter. Appropriate water management can quickly reduce the mortality of alfalfa
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35 context of changes in rainfall patterns.

37 **Introduction**

38 Alfalfa (*Medicago sativa* L.) has spread widely because of its productivity and palatability. The
39 global area planted with alfalfa is about 3.2×10^7 hm^2 and is mainly distributed in the United

40   States, Russia and Argentina (*Russelle, 2001*). The area planted with alfalfa exceeds 4×10^6 hm^2
41   in China (*He, 2011*), where it is mainly distributed in the high latitudes. There has been frequent
42   crop failure due to winter conditions in recent years, such as the "Easter freeze" of 2007 in the
43   United States *(Augspurger, 2009)* and the frost in northern China from 2012 to 2020 (*Yang,*
44   *2019*). The failure of alfalfa to overwinter not only causes huge economic losses (*Castonguay,*
45   *2006*), but also reduces biological nitrogen fixation and increases nitrous oxide emissions from
46   agronomic ecosystems, leading to an increased risk of global warming (*Crews & Peoples, 2004*;
47   *Robertson, 2000*). The IPCC Fifth Assessment Report showed that the average temperature of
48   the world had risen by 0.85°C in the past 100 years and by 1°C in China over the past few
49   decades (*Fang, 2018*). Two major factors linked to climate change are likely to affect plant
50   winter survival: changes in precipitation and temperatures (*Be'langer, 2002*; *CCAF, 2001*).
51   Extreme low temperature is more likely to reduce the survival rate of alfalfa in winter owing to
52   reduced snowfall, and greater temperature fluctuation can make alfalfa break dormancy
53   prematurely, exposing vulnerable buds to subsequent killing frost and causing sustained
54   damage (*Augspurger, 2009*). Modeling of global climate change has predicted that alfalfa death
55   due to reduced snowfall and greater temperature fluctuation will occur more frequently in the
56   future (*Carol, 2013*; *Ji, 2017*).

57   Alfalfa needs to undergo a period of low temperature and a short photoperiod to obtain its
58   freezing tolerance, and this is known as cold acclimation (*Theocharis, 2012*; *Trischuk, 2014*).
59   Appropriate cultivation measures are also an effective way to improve cold resistance. Water
60   plays an important role in the winter hardiness of alfalfa because freezing injury is mainly caused
61   by cell dehydration (*Xu, 2020a*; *Xu, 2020b*; *Zhang, 2015*). Water can not only affect the cold
62   resistance of alfalfa by changing the morphology and spatial distribution of the root system, but
63   also protect cells from low-temperature damage through physiological metabolic
64   pathways (*Castonguay et al., 2006*). In addition to precipitation amount, the impact on root
65   growth also includes precipitation timing and interval. Research has shown that precipitation
66   events have different effects on various plants, and high-frequency light precipitation events
67   were found to have a greater impact on herbaceous plants *(Schwinning & Sala, 2004;*
68   *Schwinning, 2003)*. The root system is key to the ability of alfalfa to resist low temperature.
69   Plants can regulate root system development in response to dynamic changes in soil
70   moisture (*Comas, 2013*; *Li, 2020*). Previous studies have documented that below-ground biomass
71   (BGB) (*Larson & Smith, 1963*; *Liu, 2015*), root crown (*Larson & Smith, 1963*; *Liu et al., 2015*;
72   *Schwab, 1996*), lateral roots (*Liu et al., 2015*; *Smith, 1951*) and root system spatial
73   distribution (*Castonguay et al., 2006*) all affect the cold resistance of alfalfa. According to the
74   cross acclimation theory *(Kong & Henry, 2019a; 2019b)*, low-frequency heavy precipitation will
75   increase the time plants spend in drought conditions, and so improve the cold resistance of alfalfa.
76   This is because drought can enhance the antioxidant capacity and osmotic regulation of plants,
77   including changes in malondialdehyde (MDA) (*Schwab et al., 1996*), proline (Pro) (*Janska,*
78   *2010*), soluble sugars (SS) (*Trischuk et al., 2014*) and starch content (*Xu et al., 2020a*), and these
79   are closely related to the cold resistance of alfalfa. MDA reflects the degree of membrane lipid

80 peroxidation in the cell membrane, and its content is directly proportional to the low temperature
81 injury of alfalfa (*Schwab et al., 1996*). Proline improves the cold resistance of plants by
82 regulating osmotic balance and increasing protein solubility (*Janska, 2010*). Soluble sugar acts as
83 osmotic regulator, cryoprotectant, and signaling molecule to stabilize the cell membrane and
84 scavenge reactive oxygen species under low temperature (*Trischuk et al., 2014*). Starch can be
85 broken down into soluble sugar to improve the cold resistance of alfalfa (*Xu et al., 2020a*).

86 Our experiment simulated the effects of different precipitation patterns on alfalfa root traits and
87 cold resistance. The purpose was to: (1) study the response of root morphology and spatial
88 distribution to different precipitation events; (2) clarify the relationship between root traits and
89 cold resistance; (3) explain the effect of different precipitation patterns on cold resistance; (4)
90 clarify whether precipitation patterns have differences in the root traits of alfalfa cultivars. These
91 are of great significance since the water management could prove important for increasing cold
92 resistance.

93

94 Materials & Methods
95 Experiment Location and Materials

96 This experiment was conducted in a controlled greenhouse at the Institute of Animal Science,
97 Chinese Academy of Agricultural Sciences (Beijing, China) from May to September 2020, with
98 25°C/20°C (day/night), 14 h/10 h (light/dark) and photosynthetic photon flux density of 350
99 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 60%–65% relative humidity. Alfalfa seeds were disinfected with sodium
100 hypochlorite (1% NaClO) for 30 min and washed with deionized water five times. We then
101 selected seeds of the same size and germinated them in a Petri dish with 14 h light and 10 h dark
102 at 25°C. After 72 h we moved three germinant seeds into one polyvinyl chloride (PVC) pipe with
103 an inner diameter of 18 cm and height of 50 cm. A nylon mesh bag was placed in each pipe (to
104 facilitate later sampling) and this was filled with 2.5 kg of sterilized dry mixture with a volume
105 ratio of 4:1 sandy soil and nutrient soil mixture. The nutrient soil mixture is a cultivation medium
106 (composed of peat moss and lime), named TS1, produced by Klasmann-Deilmann. TS1 contains
107 1.6% total nitrogen, 0.1% P2O5, 0.2% K2O (N: P: K = 14:10:18) and 91% of organic matter,
108 with a conductivity of 0.9 dS/m and pH of 5.8. The water-holding capacity (WHC) of the
109 mixture was measured to be 38.35%. One plant was left in each PVC pipe 1 week after
110 transplanting according to its height (about 15 cm), and cultivation continued for another 2
111 weeks before subsequent experimentation. The soil moisture content was kept at 60%–65%
112 WHC by weighing the pipe every second day. Weeds and pests were removed regularly.

113 Experiment Designs and Treatments

114 A randomized complete block design was used, with three alfalfa cultivars (C), three simulated
115 rainfall events (SRE) and two phases. The three alfalfa C were WL168, WL353 and WL440

116   (with a fall dormancy score of 2, 4 and 6, respectively; provided by Beijing Zhengdao Seed
117   Industry Co., Ltd.). These are commonly planted in large areas of northern China and represent
118   the range in fall dormancy scores of alfalfa C grown in the region. The three SRE were:
119   irrigation once every second day (D_2), every four days (D_4) and every eight days (D_8). D_2
120   represented a high-frequency light precipitation and D_8 represented a low-frequency heavy
121   precipitation. The two phases were phase 1 (before cold acclimation) and phase 2 (after cold
122   acclimation). The combined $3 \times 3 \times 2 \times 6$ repetitions = 108 PVC pipes.

123   According to the preliminary test, the soil moisture content of D_2, D_4 and D_8 before the next
124   irrigation was about 50%, 40% and 30% of WHC respectively. We carried out three irrigation
125   interval treatments, and total irrigation quotas remained the same between different treatments
126   and determined by D_2 (keeping the soil moisture content at 60%–65% of the WHC by weighing
127   every second day). After 8 weeks we carried out phase 1 sampling ($3 \times 3 \times 6 = 54$ pipes in total,
128   as shown in Fig.1). The aboveground and underground parts were separated and then carefully
129   removed from the nylon mesh bag in each PVC pipe to minimize damage to the spatial
130   distribution of the root system. The root surface mixture was washed away gently with distilled
131   water by hand and the roots were placed evenly in a transparent acrylic tray with 1200 mL of
132   distilled water. They were then scanned with a MICROTEK Scan Maker i800plus (Microtek
133   Technology Co., Ltd., Shanghai, China) with a resolution of 600 dpi. Immediately after scanning,
134   about 5 cm of the root crown was used to determine electrical conductivity and physiological
135   indicators. We divided the sample into two parts, one part was used for the determination of LT_{50},
136   the other part was used for the determination of physiological indicators. The remainder of the
137   root was measured to calculate biomass. The aboveground and underground parts were weighed
138   after being placed in an oven at 65℃ for 48 h and the dry weight were above-ground biomass
139   (AGB) and below-ground biomass (BGB).

140   The rest half of the experimental plants were moved to an LRH-200-GD low-temperature light
141   incubator (Taihong Medical Instruments, Guangdong, China) for the low-temperature
142   experiment (phase 2). The initial temperature was 25°C/20°C (day/night) with a photoperiod of
143   10 h light and 14 h dark, and the photosynthetic photon flux density was 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The
144   temperature was decreased to 5°C/0°C (day/night) at a rate of 2°C$\cdot d^{-1}$ and the light intensity was
145   decreased to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a rate of 20 $\mu mol \cdot m^{-2} \cdot s^{-1} \cdot d^{-1}$, simulating the cold adaptation
146   environment of alfalfa. Sampling was carried out after a further 72 h of cold acclimation. During
147   this phase of the experiment, plants were watered as in the previous phase. The root was
148   carefully rinsed by hand with distilled water after the test and the root crown sample was divided
149   into two parts. One part was used for the measurement of electrical conductivity and the other for
150   physiological indicators (kept at -80℃).

151   **Measurements**

152 **Root Morphological Indicators**

153 We used a Win-RHIZO 2017a (Regent Instruments, Inc., Quebec, QC, Canada) to analyze the

154 scanned images (Fig. 2). The process included thresholding, framing, editing breakpoints and

155 eliminating loops to obtain the root morphological indicators, root length, root surface area

156 (RSA), root volume (RV), root forks (RF) and average link length (ALL). Topological index (TI)

157 and fractal dimensions (FD) were obtained by calculation.

158 TI is used to reflect the spatial structure of different root systems and is defined as log altitude

159 (A)/log magnitude (M) where A is the number of links in the longest path from an exterior link

160 to the most basal link of the root system and M is the total number of the exterior links (*Bouma,*

161 *2001*). When TI is close to 0.5, the root system tends to a dichotomous branching and when close

162 to 1 it tends to herringbone branching. FD were obtained according to the box-dimension

163 method (*Bouma et al., 2001*; *Fitter, 1986*; *Fitter, 1991*; *Harrar & Hamami, 2007*).

164 **Semi-lethal Temperature**

165 The semi-lethal temperature (LT_{50}, the temperature at which the relative permeability of

166 intracellular ions attains 50%) was used to represent the cold resistance of alfalfa in this

167 study (*Anower, 2016*). We took the 5 cm underground taproot as the root crown, and then we cut

168 the root crown into 9 pieces of 2-3 mm (based on length instead of diameter), and these pieces

169 were put into nine 2-mL centrifuge tubes (*Xu, 2020a*). Tubes were placed at 8°C for 2 h. The

170 subsequent freezing test was conducted in a ZX-5C constant-temperature circulator (Zhixin

171 Instrument, Shanghai, China) under a decreasing series of nine temperatures, and tubes with

172 samples were kept in alcohol for 1.5 h at each temperature. The temperatures of the alcohol were

173 different due to the difference between the LT_{50} under the two treatments. For the samples

174 collected in phase 1, nine temperatures were set to 8, 6, 4, 2, 0, −2, −4, −6 and −8°C. For the

175 samples collected in phase 2, nine temperatures were set to 0, −2, −4, −6, −8, −10, −12, −14 and

176 −16°C. After 1.5 h at the first temperature in each phase, one tube was transferred to storage at

177 that temperature; after 1.5 h at the second temperature another tube was removed for storage at

178 that temperature; and so on until all nine tubes in each phase were stored at their designated

179 temperatures. We then removed the pieces of root crown from each 2-mL tube and placed them

180 in one 15-mL tube and added 5 mL deionized water. This tube was shaken on an HZQ-A

181 gyratory platform shaker (Hengrui Instrument and Equipment, Changzhou, China) at 120 rpm for

182 12 h at 25°C. Next used a conductivity meter FE38 (Mettler, Shanghai, China) to measure the

183 electrical conductivity as EL_1. The sample was autoclaved at 121°C for 30 min and, on

184 remeasuring, its electrical conductivity was found to be EL_2. The electrical conductivity of

185 deionized water was EL. Relative electrolyte leakage can be calculated according to Eq. (1) and

186   the semi-lethal temperature can be calculated by logistic Eq. (2). In Eq. (2), x is the freezing

187   temperature, y is the relative electrical leakage and A, B and k are constants:

188   Relative electrolyte leakage (%) = $(EL_1\text{-}EL)/(EL_2\text{-}EL) \times 100$.   (1)

189   $y = A/(1+B \times e^{-kx}) \times 100\%$.   (2)

Root Physiological Indexes

191   Physiological indexes were also measured on root crown of alfalfa. Ground the other part of the

192   crown sample kept at -80°C into powder and then determined their SS (*Buysse & Merckx, 1993*),

193   starch (*Buysse & Merckx, 1993*), MDA (*Dhindsa, 1981*) and Pro content (*Bates, 1973*).

194   Malondialdehyde (MDA)

195   A 0.2-g sample was placed in a 10-ml test tube for determination of MDA. (1) Added 5 mL 0.1%

196   cold trichloroacetic acid (TCA) and then the homogenate was centrifuged at 10000 r/min for 5

197   min at 4 °C. (2) To 1 mL aliquot of the supernatant 4 mL 20% TCA containing 0.5% TBA were

198   added. The mixture was heated at 95°C for 20 min and then quickly cooled in an ice-bath. After

199   centrifuging at 4000 r/min for 15 min, then 2 mL supernatant was removed to a cuvette and 2 mL

200   deionized water used as control. (3) The absorbance of the supernatant was measured at 450 nm

201   (OD_{450}), 532 nm (OD_{532}) and 600 nm (OD_{600}). The MDA content was measured according to Eq.

202   (3), where V_1 (mL) is the total volume of the supernatant, V_2 (mL) is the volume of the

203   measurement and DW (g) is the weight of the freeze-dried sample:

204   MDA (nmol/g DW) = $[6.452 \times (OD_{532} - OD_{600}) - 0.559 \times OD_{450}] \times V_1/(V_2 \times DW)$.   (3)

205   Proline (Pro)

206   A 0.2-g sample was placed in a 10-ml test tube for Pro determination. (1) Added 5 mL of 3%

207   aqueous sulfosalicylic acid solution and then transferred the sample to a boiling water bath for 20

208   min and obtained the Pro extraction after cooling. (2) A 2-mL extraction was moved to another

209   test tube, 2 mL of glacial acetic acid and 2 mL 2.5% of acidic ninhydrin solution were added and

210   the extraction was then transferred to a boiling water bath for 60 min. (3) Added 4 mL of

211   methylbenzene and the tube was shaken after cooling before being centrifuged at 5000 r/min for

212   5 min. (4) Measured the absorbance of the supernatant at 520 nm and calculated the Pro content

213   according to the standard curve and Eq. (4), where C is the Pro content obtained from the

214   standard curve, V_1 (mL) is the total volume of the extraction, A (mL) is the volume of the

215   measurement and DW (g) is the weight of the freeze-dried sample:

216   Pro (μg/g DW) = $(C \times V_1/A) / DW$.   (4)

217   Soluble sugar (SS)

218   A 0.2-g sample was placed in a 50-mL test tube for determination of the soluble sugar. (1)

219   Added 20 mL deionized water and then transferred the tube to a boiling water bath for 20 min. (2)

220   The tube was centrifuged at 3500 r/min for 10 min after cooling and the supernatant transferred

221   to a volumetric flask and diluted to 100 mL as an extraction solution (the residue was used later

222   to determine starch content). (3) Placed 1 mL of the extraction solution in another test tube and

223   added 4 mL 2% anthrone ethyl acetate, then placed the tube in a water bath at 90°C for 15 min.

224   (4) After the tube had cooled and measured the absorbance of the extraction solution at 625 nm.

225   The content of soluble sugar was calculated according to the standard curve and Eq. (5), where C

226   (μg) is the soluble sugar content according to the standard curve, V_1 (mL) is the total volume of

227   the extraction, A (mL) is the volume of the measurement and DW (g) is the weight of the freeze-

228   dried sample:

229   Soluble sugar (%) = $(C \times V_1) / (A \times DW \times 10^6) \times 100$.   (5)

230   Starch

231   The remaining residue was moved to a 20-mL test tube for determination of the starch. (1)

232   Added 8 mL of hydrochloric acid and boiled the tube in a water bath for 45 min, then transferred

233   the contents into a volumetric flask and added 8 mL sodium hydroxide and diluted it to 50 mL.

234   (2) Placed 1 mL of the supernatant in a volumetric flask and diluted it to 25 mL as an extraction

235   solution. (3) Added 4 mL of anthrone to 1 mL extraction solution and placed the tube in a boiling

236   water bath for 5 min. (4) After cooling, the absorbance was measured at 625 nm. Starch content

237   was calculated according to the standard curve and Eq. (6), where C (μg) is the starch content

238   according to the standard curve, V (mL) is the total volume of the extraction solution, A (mL) is

239   the volume of the measurement and DW (g) is the weight of the freeze-dried sample:

240   Starch (%) = $C \times V \times 0.9 / (A \times DW \times 10^6) \times 100$.   (6)

241   **Statistical Analyses**

242

243   Shapiro-Wilk test and Levene test showed that all data in this experiment obeyed a normal

244   distribution and satisfied the homogeneity of variance. Data in this study were subjected to a

245   two-way analysis of variance between treatments using SPSS 20.0 (SPSS Inc., Chicago, IL,

246   USA). Multiple range tests were performed using least significant differences, and differences

247   were considered significant at $p < 0.05$ and $p < 0.01$; ns was not significant. Principal component

248   analysis (PCA) was also conducted in this experiment.

249   **Results**

250   **Biomass**

251   After 8 weeks, BGB and the ratio of BGB to AGB (R/S) growth differed significantly ($p < 0.01$)

252   among SRE; nevertheless, there was no significance ($p > 0.05$) in AGB (Table 1). As rainfall

253   intervals increased, BGB and R/S showed an increasing trend. D_8 had the highest BGB and R/S

254   (1.01 g and 0.68, respectively) and these were significantly higher than those of D_2. There was

255   no major impact on AGB and R/S ($p > 0.05$) of the three C within the same water treatment

256   (Table 1). The BGB of WL440 was 0.64 g, which was significantly ($p < 0.01$) lower than that of

257   WL168 and WL353. Further analysis showed significant interactions between SRE and C on R/S,

258   and the former were found to have played a more important role (Table 2).

259   **Root Morphology**

260   SRE had a little effect ($p > 0.05$) on the primary root length (PRL) of alfalfa (Table 3). D_2 had

261   the longest lateral root length (LRL, 247 cm) followed by D_4 and D_8. The crown diameters (CD)

262   of D_4 and D_8 were 4.21 and 4.27 mm, respectively, significantly ($p < 0.05$) wider than that of D_2.

263   RSA varied significantly ($p < 0.01$) among the three SRE; D_8 had the minimum at 46.0 cm^2. D_4

264   and D_8 had the same RV, 0.81 cm^3, significantly ($p < 0.01$) larger than that of D_2. Specific root

265   length (SRL) was significantly ($p < 0.01$) inversely proportional to rainfall events. D_2 had the

266   greatest SRL, 452 cm/g, followed by D_4 (335 cm/g) and D_8 (221 cm/g). The three alfalfa C

267   differed significantly ($p < 0.01$) in LRL, but not in PRL (Table 3). WL168 showed a significantly

268   ($p < 0.01$) shorter LRL than WL440 and WL353. There was no evidence of a significant

269   difference in CD and RSA between various C. The RV of WL168 and WL353 were both 0.80

270   cm^3, a value significantly ($p < 0.01$) greater than that of WL440. The SRL of WL168 and

271   WL353 were 277 and 278 cm/g, respectively, much lower than that of WL440. In every

272   treatment combination (SRE × C), the effect of rainfall events on root surface area and specific

273   root length was greater than that of C (Table 2).

274   **Root System Architecture**

275   Basic information about the root system architecture in phase 1 is shown in Table 4. SRE had

276   major effects ($p < 0.01$) on RF, notably on D_2 at 1572. The ALL of D_8 was 0.136 cm, which was

277   significantly longer than that of D_2 and D_4. Across the three SRE, the TI ranged from 0.600 to

278   0.634 and FD ranged from 1.390 to 1.469. The TI and FD showed a reverse trend. A significant

279   ($p < 0.01$) effect was detected for RF and ALL in various alfalfa C, but there was no significant

280   ($p > 0.05$) difference in TI and FD. WL353 had the maximum RF, 1390, which was significantly

281   more than RF in WL168 and WL440. In contrast, the ALL of WL353 was 0.117 cm, which was

282   shorter than that of WL440. There were no major interactions between SRE and C in these four

283   indicators (Table 4).

284   **Semi-lethal Temperature**

285   We observed that the cold resistance of alfalfa that had not undergone cold acclimation was

286   relatively weak (Fig. 3). From phase 1 to phase 2 the LT_{50} of three SRE decreased by an average

287   of 8.4°C, and D_2 had the largest decline at 6.1°C. D_8 had the lowest LT_{50} in both phases (0.32

288   and −6.5°C, respectively). Simultaneously, there were also significant ($p < 0.01$) differences in

289   the LT_{50} in the two phases among the three alfalfa C. The LT_{50} of WL168 decreased the most

290   and reached 6.9°C, followed by WL440 and WL353 (5.9 and 5.8°C, respectively). WL168 had

291   the greatest cold resistance among the three C in phase 2, and its LT_{50} was −6.8°C.

292   Principal component analysis (PCA, Kaiser-Meyer-Olkin (KMO) value was 0.728 and $p < 0.01$)

293   of 10 variables was used to identify the correlations between the variables and LT_{50}, which were

294   associated with the first two principal components (Fig. 4). Different colors denote different SRE

295   and shapes refer to cultivars, and SREs play a more important role than cultivars in the

296   difference of root traits. PCA axis 1 primarily reflected the morphological and spatial

297   characteristics of root systems (LRL, RF and TI), which accounted for 37.56% of the overall

298   variance in the standardized variables. Axis 2 mainly reflected the biomass of the alfalfa,

299   explaining 16.82% of the standardized variance (Table 5). BGB, PRL and RSA were positively

300   correlated with CD, RV and FD, respectively. LRL was negatively correlated with BGB, CD and

301   RV; and TI was negatively correlated with RSA, RF and FD. LT_{50} was positively correlated with

302   LRL, RSA, FD and RF, but inversely correlated with BGB, CD, RV and TI. Lateral root length,

303   root forks and fractal dimension may have a greater contribution to the difference in LT_{50}

304   between plants (Fig. 3).

305   **Physiological Indicators**

306   Compared with phase 1, the MDA content showed an increasing trend in phase 2, and there were

307   significant ($p < 0.01$) differences among various rainfall events (Fig. 5). The content of MDA of

308   D_8 increased by 25% from phase 1 to phase 2, which was less than that of D_4 (38%) and D_2

309   (59%). There were no major changes in MDA content between the three C at 25°C/20°C

310   (day/night), but a significant difference appeared after the low-temperature experiment, and

311   WL168 had a lower MDA content (100.9 nmol·g^{-1} DW).

312   The Pro content of the three rainfall events showed significant ($p < 0.01$) differences in the two

313   phases (Fig. 6), but there was no significant difference among the three C. D_8 had the highest Pro

314   content at 2.61 and 3.26 mg· g^{-1} DW in phase 1 and phase 2, respectively, followed by D_4 (1.06

315   and 1.26 mg· g^{-1} DW) and D_2 (0.31 and 0.83 mg· g^{-1} DW).

316   The soluble sugar content of the three treatments increased after cold acclimation (Fig. 7). In the

317   same phase, SRE had a significant ($p < 0.01$) impact on the soluble sugar content of alfalfa root,

318   which was manifested as an increasing trend as the rainfall interval increased. There were major

319   effects ($p < 0.01$) on the soluble sugar content of the two phases among the three alfalfa C.
320   WL440 had 118 and 127 mg· g^{-1} DW soluble sugar content in phase 1 and phase 2, respectively,
321   significantly higher than those of WL353 and WL168.

322   The starch content showed a downward trend at phase 2 for the three rainfall events in all three C
323   (Fig. 8). In the two within-phase comparisons, SRE had a significant ($p < 0.01$) impact on the
324   soluble sugar content of alfalfa roots, while the choice of C did not. The starch content of D_8 was
325   95.4 and 68.5 mg· g^{-1} DW in phase 1 and phase 2, respectively, which was higher than in the
326   other two treatments.

327

328   **Discussion**
329   **Root Biomass**

330   Swemmer (*2007*) and Heisler-White (*2008*) demonstrated that rainfall interval and rainfall
331   amount are the key factors affecting the allocation of plant biomass. Rainfall events had no
332   significant effect on the AGB of alfalfa, while longer rainfall intervals increased BGB and R/S
333   (Table 1). This allometric growth relationship between root and shoot is an adaptive strategy by
334   plants to various soil water conditions (*Padilla, 2009*). Research has shown that longer rainfall
335   intervals can enhance the drought resistance of plants by promoting root growth and increasing
336   energy distribution to underground parts (*Den Herder, 2010*; *Jangpromma, 2012*; *Padilla et al.,*
337   *2009*). There was little difference in AGB between the three C, but the C with lower fall
338   dormancy level had higher BGB and R/S. Root biomass has been proven to be an indicator of
339   stress resistance (*Bloor, 2018*), and the C with a lower level of fall dormancy distributes more
340   energy to its roots. This also explains, to a certain extent, why alfalfa with a lower fall dormancy
341   score has better cold resistance (*Oppelt, 2001*; *Xu et al., 2020b*).

342   **Root Morphological Traits and Architecture**

343   Root plasticity determines the ability of plants to survive in the ever-changing soil
344   environment (*Tian, 2014*). The primary root mainly plays a role of fixing, storing and
345   transporting substances. It has a longer lifespan and slower metabolism, and there are no
346   significant differences in response to various rainfall events (*Gruber, 2013*). Lateral roots are the
347   main parts of the root system that absorb water and nutrient; they have a shorter lifespan,
348   stronger metabolism and lower resistance to abiotic stress, and are more sensitive to changing
349   environments (*Li et al., 2020*; *Padilla et al., 2009*; *Withington, 2006*). Through research on rice
350   (*Oryza sativa* L.) and arabidopsis (*Arabidopsis thaliana* L.), *Pedersen* (*2021*) found that a longer
351   rainfall event results in an overall altered root system that ranges from changes in root system
352   architecture, including fewer lateral roots and thicker primary root (*Hodge, 2004*; *Schwab et al.,*

353 *1996*). Larger root surface area and specific root length are beneficial to the root water
354 absorption efficiency system (*Hassouni, 2018*), but attention should be given to the cost of water
355 absorption under abiotic stress. Lateral roots are the most active part of the entire root system,
356 and their faster turnover rate also increases the consumption of stored substances (*Guo, 2004*;
357 *Rewald, 2011*; *Withington et al., 2006*). D_8 has the shortest lateral root length (Table 3) and less
358 consumption of stored substances, which can explain why alfalfa grown in intervals of longer
359 rainfall showed better cold resistance in a subsequent low-temperature test (Fig. 2). Features
360 such as a greater root volume and a small root surface area are evident after a longer rainfall
361 interval, which enables plants to cope better with unpredictable soil conditions (*Pedersen et al.,*
362 *2021*). Genotypes play an important role in the growth of plant roots under the same environment
363 conditions and cultivation measures. Root systems of different cultivars respond differently to
364 change in soil moisture (*Tron et al., 2015*). A field study was conducted to analyze the root
365 system development ability of nine alfalfa cultivars; root biomass, primary root, lateral root, root
366 surface area and root crown were significantly different among cultivars (*Zhang et al., 2002*).
367 The three C presented various root trait responses to rainfall events: alfalfa with a low level of
368 fall dormancy had shorter lateral roots and specific root length and a larger root volume,
369 consistent with the results found by *Filippo* (*2010*). The spatial structure of a root system can
370 further describe the distribution of roots in the soil. A study of the effects of rainfall on various
371 species found that rainfall events have important effects on root system architecture, including in
372 the roots of herbaceous plants (*Kume, 2006*). Root forks and average link length reflect the
373 branching of plant roots. Root forks are related to water absorption efficiency *(Bauhus, 2000)*
374 and average link length represents the space expansion ability of the root system (*Walk, 2004*).
375 There is a negative correlation between root forks and average link length, and this depends on
376 the soil conditions *(Schenk & Jackson, 2002a; 2002b)*. The results of rainfall events on alfalfa
377 show that a root system under a longer rainfall interval has a longer average link length, while
378 more root forks appear under shorter rainfall intervals (*Kong, 2014*). Root systems with a larger
379 number of forks are more advantageous in resource-rich soil because they can quickly occupy
380 the space available for rapid growth. Those with a longer average link length can improve
381 competitiveness under water shortage because root overlap and unnecessary internal competition
382 is reduced (*Bauhus et al., 2000*; *Enquist & Niklas, 2002*; *Guswa, 2010*). We verified this trade-
383 off relationship between root forks and average link length in the three C (Table 4). Topological
384 index and fractal dimensions are both parameters that describe the root system architecture. Low-
385 frequency heavy precipitation events are conducive to the development of herringbone branching
386 in a root system (Table 4). Researcher has divided the branching patterns into dichotomous and
387 herringbone according to the two extreme values of the TI, although the branching pattern of
388 most plants falls between the two types (*Fitter, 1986*). A root system tends to dichotomous

389 branching when the plant is in high-nutrient soil and the TI is close to 0.5; the branching pattern
390 tends to herringbone when resources are scarce and the TI is close to 1 (*Fitter & Stickland, 1992*;
391 *Glimskär, 2000*; *Li et al., 2020*; *Lynch, 2019*). Fractal dimensions are also important parameters
392 in explaining the spatial structure of the root system. The change trend in fractal dimensions and
393 root forks is consistent in describing the use of space by roots, and our results agreed with those
394 of *Li* (*2020*) and *Margitta* (*2005*). *Tron et al.* (*2015*) modeled the transpiration of 48 root
395 architecture types under 16 drought scenarios and different soil structures and textures; they
396 reported that root architecture did not fully explain plant water use and suggested relating
397 specific root architecture with genotype and other characteristics. Genotypes differ in their
398 localization of root biomass at different depths under water stress condition; tolerant genotypes
399 produce deeper and more vigorous roots in the search for water (*Faroop et al., 2019*). *Manschadi*
400 *et al.* (*2006*) studied root architectural traits in the adaptation of wheat to water-limited
401 conditions using a drought-tolerant and drought-susceptible genotype; the tolerant genotype
402 developed a compact vertical root system allowing it to extract less water during early growth
403 stages but more as the crop matured. WL168 has the smallest root forks and average link length,
404 and this is consistent with the study of *Faroop* (*2019*).

405 **Semi-lethal Temperature and Root Traits**

406 Alfalfa has to undergo a low-temperature and short-photoperiod process to maximize its cold
407 resistance (*Theocharis et al., 2012*), and this characteristic is shown in all three
408 cultivars *(Trischuk et al., 2014)*. Whether at normal temperature or undergoing low-temperature
409 stress, the semi-lethal temperature of D_8 was significantly lower than that of the other two (Fig.
410 2). Simultaneously, the cross acclimation of drought and low temperature has been confirmed in
411 creeping bentgrass (*Agrostis stolonifera* L.) (*Zhang et al., 2015*) and alfalfa (*Xu et al., 2020b*),
412 which provides theoretical support for improving cold resistance of alfalfa by water management.
413 To further understand the relationship between water and cold resistance, we conducted a PCA
414 analysis among root system traits and semi-lethal temperature (Fig. 4). The positive correlation
415 factors that affect cold resistance of alfalfa mainly include below-ground biomass, crown
416 diameter, root volume and topological index, while the increase in lateral root length, root
417 surface area, root forks and fractal dimensions reduced the semi-lethal temperature. Some of our
418 conclusions contradicted previous research (*Johnson, 1996*; *Larson & Smith, 1963*; *Liu et al.,*
419 *2015*; *Smith, 1951*), but these differences were mainly caused by environmental factors rather
420 than by low-temperature stress. The root crown is the most sensitive part of the root system to
421 temperature changes and is crucial to overwintering and regeneration (*Bélanger, 2006*; *Janska et*
422 *al., 2010*). The plasticity of the root crown is an important strategy for alfalfa in its adaptation to
423 the cold climate in northern regions. As the crown diameter increases, the cold resistance of

424   alfalfa is gradually enhanced (*Liu et al., 2015*; *Schwab et al., 1996*). The root biomass is related

425   to the accumulation of organic matter and the herringbone branching is conducive to alfalfa's

426   absorption of deeper water in cold winter, which contribute to improving the plant's cold

427   resistance (*Larson & Smith, 1963*; *Viands, 1988*). Longer lateral roots accelerate nutrient

428   consumption and are more susceptible to freezing under low-temperature stress (*Schwab et al.,*

429   *1996*; *Withington et al., 2006*). Studies have confirmed that low-temperature stress inhibits plant

430   growth (*Liu, 2019*; *Venzhik, 2011*), but little is known about how these characteristics affect cold

431   resistance. The above analysis demonstrates the regulatory effect of rainfall events on root

432   system traits, and the next step is to manipulate these traits to enhance plant stress tolerance. It is

433   possible that gene editing technology may allow plant root traits to be changed and so permit

434   better adaptation to low-temperature environments (*Nakamichi, 2016*).

435   **Root Physiological Indexes**

436   In winter, physiological regulation such as osmotic regulation, antioxidant regulation and

437   induction of antifreeze gene expression is the most important way for plants to adapt to low-

438   temperature stress (*Bertrand, 2017*; *Choudhury, 2017*; *Theocharis et al., 2012*). Research has

439   concluded that it is a significant correlation between cold resistance and the cell membrane, and

440   plants with stronger cold resistance have a lower phase-transition temperature (*Anower et al.,*

441   *2016*). MDA is the final product of cell membrane lipid redox, which can destroy the structure

442   and function of proteins, nucleic acids and polysaccharides. Plants produce a larger amount of

443   MDA under low-temperature stress, and its content has a significant negative correlation with the

444   freezing tolerance of alfalfa (*Choudhury et al., 2017*; *Xu et al., 2020a*). In addition, excessive

445   reactive oxygen species (ROS) can also affect cell activity. To reduce ROS damage, cells

446   maintain their integrity through osmotic regulation (mostly of Pro, soluble sugar and

447   starch) (*Anower et al., 2016*; *Bertrand et al., 2017*; *Castonguay, 2011*). Pro can act not only as an

448   osmotic regulator or ROS scavenger, but also as a molecular chaperone to prevent cells from

449   being damaged by low temperature (*Castonguay et al., 2011*; *Kishor, 2005*; *Nakashima, 1998*).

450   Studies have proven that soluble sugar and starch are closely related to the freezing tolerance of

451   alfalfa (*Anower et al., 2016*; *Castonguay et al., 2006*). Soluble sugar can improve the survival

452   rate of alfalfa in winter in two ways: (1) it can act as an osmotic substance and cryoprotectant to

453   lower the cell freezing point (*Castonguay, 2013*); and (2) it is a signal molecule that initiates a

454   series of cold response mechanisms (*Bertrand et al., 2017*). Starch can be hydrolyzed into

455   soluble sugar to improve the cold tolerance of alfalfa (*Sengupta, 2015*). In phase 2, the Pro and

456   soluble sugar content of D_8 was still higher than that of the other two cultivars, which indicated

457   that cold acclimation would maximize the cold resistance of alfalfa, but also that a longer

458   irrigation interval can accelerate this process (*Xu et al., 2020a*; *Zhang et al., 2015*).

459

Conclusions

461 In our study, a low-frequency heavy precipitation significantly enhanced the cold resistance of
462 alfalfa compared with a high-frequency light precipitation. The increase in root biomass and
463 crown diameter, and the decrease of lateral root length and root surface area may have
464 contributed to the difference in LT_{50} among plants receiving the different treatments. A fewer
465 root forks and a bigger topological index may reduce the redundant consumption of the root
466 system, which is beneficial to improve the cold resistance of alfalfa in a low temperature
467 environment. Alfalfa with high fall dormancy grade has more below-ground biomass and less
468 lateral root, and these characteristics are conducive to rapid acquisition of cold resistance.
469 Simultaneously, a longer irrigation interval facilitated the accumulation of proline and soluble
470 sugar content. These conclusions provide support for winter water management of alfalfa and
471 selection of varieties in areas of high latitude.

472

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478 ## Competing Interests

479 The authors declare there are no competing interests.

480 ## Author Contributions

481 Zhensong Li conceived and designed the experiments, performed the experiments, analyzed the
482 data, prepared figures and tables, authored or reviewed drafts of the paper, and approved the final
483 draft.
484 Liqiang Wan conceived and designed the experiments, reviewed drafts of the paper, and
485 approved the final draft.
486 Shuo Li performed the experiments, prepared figures and tables, and reviewed drafts of the paper.
487 Xianglin Li conceived and designed the experiments, and approved the final draft.
488 Feng He and Zongyong Tong designed the experiments, analyzed the data, and prepared figures
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709

Above-ground biomass, below-ground biomass and the ratio of below-ground biomass to above-ground biomass among simulated rainfall events or cultivars in phase 1.

Note: Mean values (n = 18) ± standard errors of the mean are shown. Different letters represent a significant difference under various SRE and C; ns indicates the difference is not significant; and $p < 0.05$ and $p < 0.01$ indicate significant difference at 0.05 and 0.01 levels, respectively. AGB, above-ground biomass; BGB, below-ground biomass; R/S, the ratio of BGB to AGB; SRE, simulated rainfall events; C, cultivars.

1   **Table 1 Above-ground biomass, below-ground biomass and the ratio of below-ground**
2   **biomass to above-ground biomass among simulated rainfall events or cultivars in phase 1.**

Treatments		AGB (g)	BGB (g)	R/S
SRE	D_2	1.60±0.26	0.64±0.25[b]	0.45±0.14[b]
	D_4	1.74±0.34	0.87±0.30[a]	0.51±0.14[b]
	D_8	1.58±0.23	1.01±0.27[a]	0.68±0.17[a]
	p value	ns	＜0.01	＜0.01
C	WL168	1.68±0.19	0.95±0.32[a]	0.57±0.21
	WL353	1.76±0.24	0.93±0.23[a]	0.56±0.12
	WL440	1.47±0.35	0.64±0.20[b]	0.48±0.16
	p value	ns	＜0.01	ns

3   Note: Mean values (n = 18) ± standard errors of the mean are shown. Different letters represent a
4   significant difference under various SRE and C; ns indicates the difference is not significant; and
5    $p < 0.05$ and $p < 0.01$ indicate significant difference at 0.05 and 0.01 levels, respectively. AGB,
6   above-ground biomass; BGB, below-ground biomass; R/S, the ratio of BGB to AGB; SRE,
7   simulated rainfall events; C, cultivars.

Interaction and simple effect analysis of simulated rainfall events and cultivars on biomass, root traits and LT_{50} among in phase 1.

Note: Significance (Sig.) and partial eta squared (PES) are shown, and ns indicates the difference is not significant. - indicates there was no significant interaction between simulated rainfall events and cultivars. PRL, primary root length; LRL, lateral root length; CD, crown diameter; RSA, root surface area; RV, root volume; SRL, specific root length; RF, root forks; ALL, average link length; TI, topological index; FD, fractal dimensions.

1   **Table 2 Interaction and simple effect analysis of simulated rainfall events and cultivars on**
2   **biomass, root traits and LT$_{50}$ among in phase 1.**

	SRE×C	SRE		C	
	Sig.	Sig.	PES	Sig.	PES
ABG	ns	-	-	-	-
BGB	ns	-	-	-	-
R/S	<0.05	<0.01	0.250	0.113	0.085
PRL	ns	-	-	-	-
LRL	ns	-	-	-	-
CD	ns		-	-	-
RSA	<0.05	<0.01	0.452	0.074	0.101
RV	ns	-	-	-	-
SRL	<0.01	<0.01	0.414	<0.01	0.349
RF	ns	-	-	-	-
ALL	ns	-	-	-	-
TI	ns	-	-	-	-
FD	ns	-	-	-	-
LT$_{50}$-phase1	ns	-	-	-	-
LT$_{50}$-phase2	ns	-	-	-	-

3   Note: Significance (Sig.) and partial eta squared (PES) are shown, and ns indicates the difference
4   is not significant. - indicates there was no significant interaction between simulated rainfall
5   events and cultivars. PRL, primary root length; LRL, lateral root length; CD, crown diameter;
6   RSA, root surface area; RV, root volume; SRL, specific root length; RF, root forks; ALL,
7   average link length; TI, topological index; FD, fractal dimensions.

Root morphological traits among simulated rainfall events or cultivars in phase 1.

Note: Mean values (n = 18) ± standard errors of the mean are shown. Different letters represent a significant difference under various SRE and C; ns indicates the difference is not significant; and $p < 0.05$ and $p < 0.01$ indicate significant differences at 0.05 and 0.01 levels, respectively.

1 **Table 3 Root morphological traits among simulated rainfall events or cultivars in phase 1.**

Treatments		PRL (cm)	LRL (cm)	CD (mm)	RSA (cm^2)	RV (cm^3)	SRL (cm/g)
SRE	D$_2$	42.40±2.5	247±15^a	3.76±0.75^b	66.3±9.9^a	0.74±0.06^b	452±109^a
	D$_4$	43.46±2.9	214±11^b	4.21±0.59^a	57.3±9.5^b	0.81±0.07^a	335±67^b
	D$_8$	43.28±3.6	162±11^c	4.27±0.56^a	46.0±9.5^c	0.81±0.04^a	221±48^c
	p value	ns	<0.01	<0.05	<0.01	<0.01	<0.01
C	WL168	42.49±2.6	185±17^b	3.88±0.76	57.8±3.6	0.80±0.07^a	277±69^b
	WL353	42.98±2.9	214±18^a	4.24±0.69	59.5±5.0	0.80±0.05^a	278±49^b
	WL440	43.68±3.4	225±24^a	4.12±0.51	52.3±5.9	0.75±0.08^b	453±114^a
	p value	ns	<0.01	ns	ns	<0.01	<0.01

2 Note: Mean values (n = 18) ± standard errors of the mean are shown. Different letters represent a
3 significant difference under various SRE and C; ns indicates the difference is not significant; and
4 *p* < 0.05 and *p* < 0.01 indicate significant differences at 0.05 and 0.01 levels, respectively.

Root spatial traits between simulated rainfall events or cultivars in phase 1.

Note: Mean values (n = 18) ± standard errors of the mean are shown. Different letters represent a significant difference under various SRE and C; ns indicates the difference is not significant; and $p < 0.05$ and $p < 0.01$ indicate significant difference at the level of 0.05 and 0.01, respectively.

1   **Table 4 Root spatial traits between simulated rainfall events or cultivars in phase 1.**

Treatments		RF	ALL (cm)	TI	FD
SRE	D_2	1572 ± 242^a	0.119 ± 0.015^b	0.600 ± 0.020^b	1.469 ± 0.043^a
	D_4	1325 ± 275^b	0.117 ± 0.020^b	0.621 ± 0.016^a	1.444 ± 0.034^a
	D_8	854 ± 118^c	0.136 ± 0.018^a	0.634 ± 0.022^a	1.390 ± 0.042^b
	p value	<0.01	<0.05	<0.01	<0.01
C	WL168	1171 ± 273^b	0.107 ± 0.021^b	0.622 ± 0.023	1.437 ± 0.050
	WL353	1390 ± 468^a	0.117 ± 0.028^b	0.610 ± 0.023	1.447 ± 0.045
	WL440	1191 ± 303^b	0.147 ± 0.020^a	0.623 ± 0.023	1.419 ± 0.044
	p value	<0.01	<0.01	ns	ns

2   Note: Mean values (n = 18) ± standard errors of the mean are shown. Different letters represent a

3   significant difference under various SRE and C; ns indicates the difference is not significant; and

4   $p < 0.05$ and $p < 0.01$ indicate significant difference at the level of 0.05 and 0.01, respectively.

Component Matrix of the first two principal components.

Table 5 Component Matrix of the first two principal components.

	Component	
	1	2
AB	0.057	0.818
BB	-0.403	0.537
PRL	-0.121	0.195
LRL	0.826	-0.288
CD	-0.199	0.348
RSA	0.779	0.375
RV	-0.420	0.544
RF	0.910	0.095
TI	-0.624	-0.099
FD	0.746	0.387

Figure 1

Schematic diagram of experiment processing and sampling.

25/20°C and 5/0°C represent the temperature of alfalfa during normal growth and cold acclimation, respectivel.
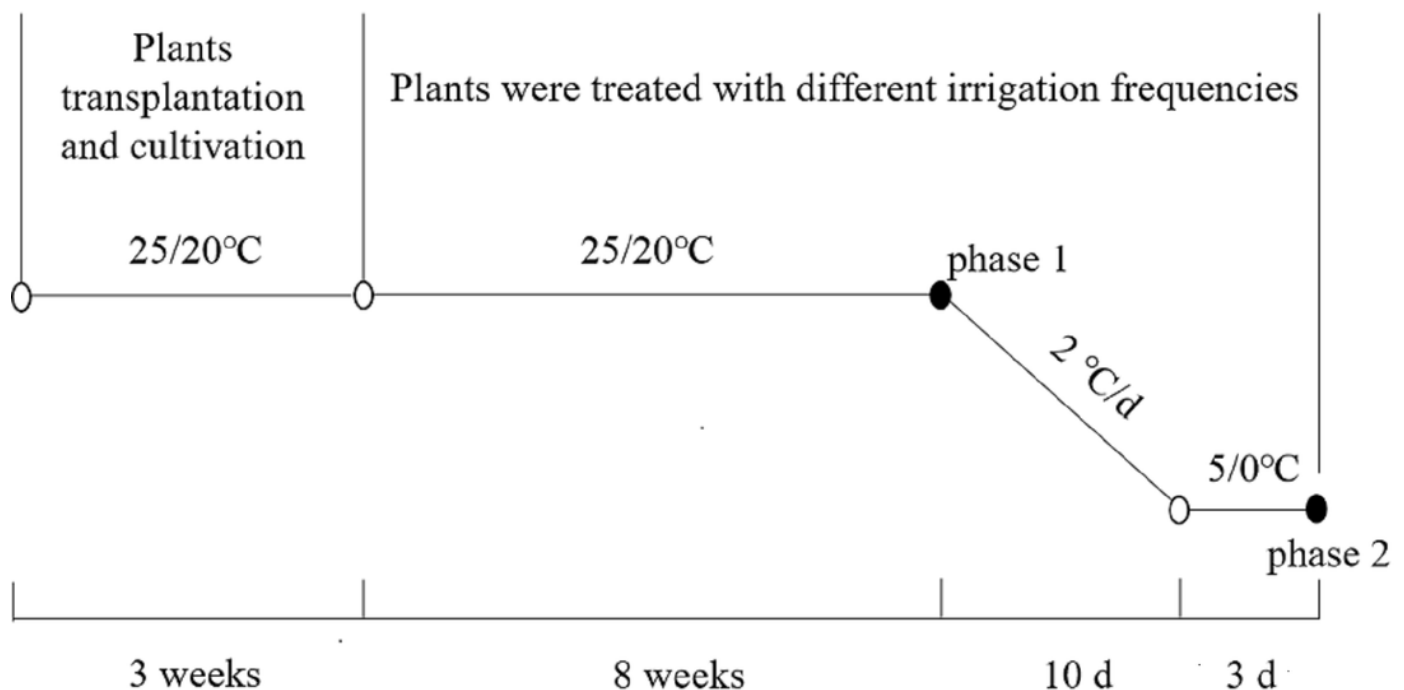
Figure 2

Scanning image of root system under three simulated rainfall events.

D_2, D_4 and D_8 represent irrigation once every second day, every four days and every eight days respectively .
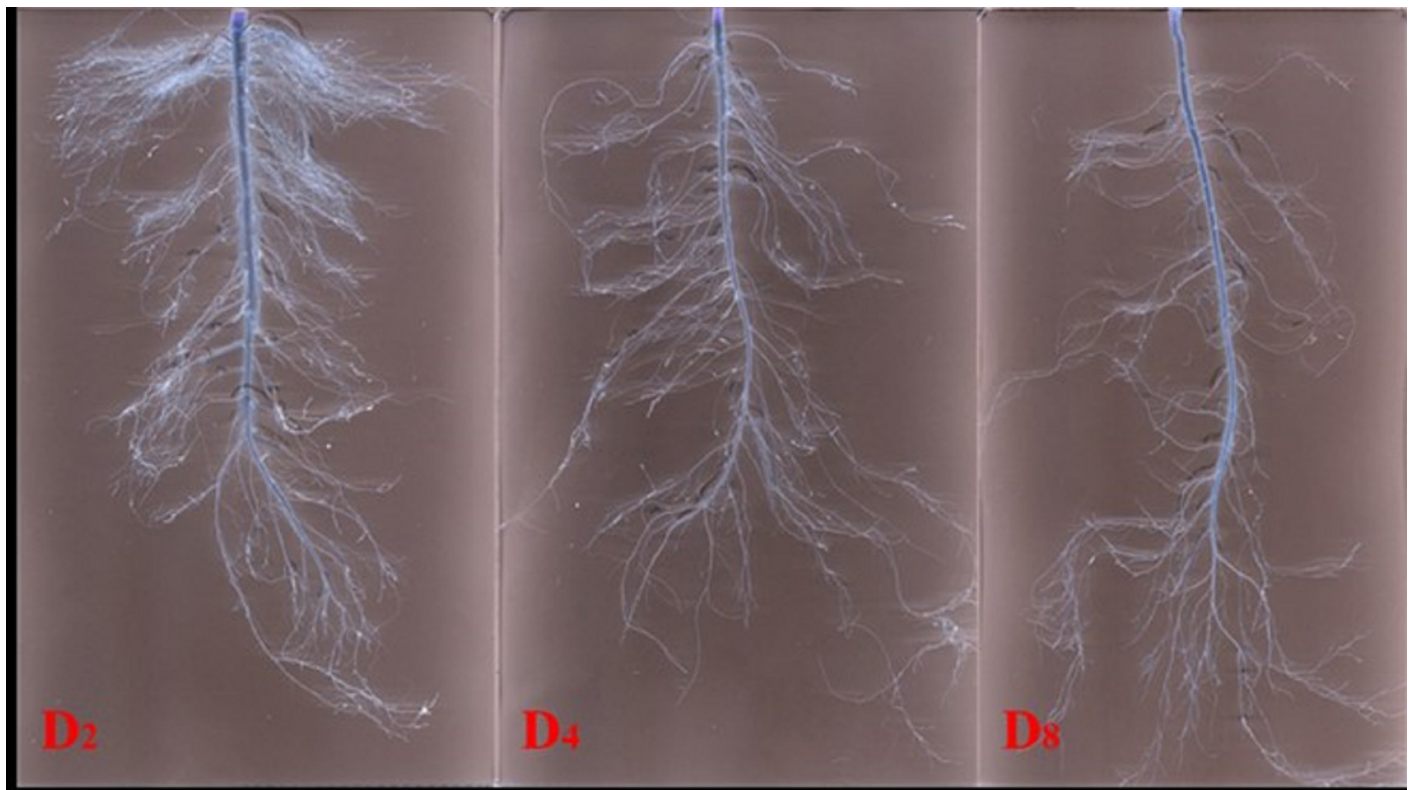
Figure 3

Semi-lethal temperatures of alfalfa cultivars crowns under different simulated rainfall events.

Mean values (n = 18) ± standard errors of the mean are shown. Different capital letters and lowercase letters indicate a significant difference between three simulated rainfall events and cultivars at the same phase at 0.01 and 0.05 level respectively; ** indicates that the same treatment had a significant difference in the two phases at 0.01 level.
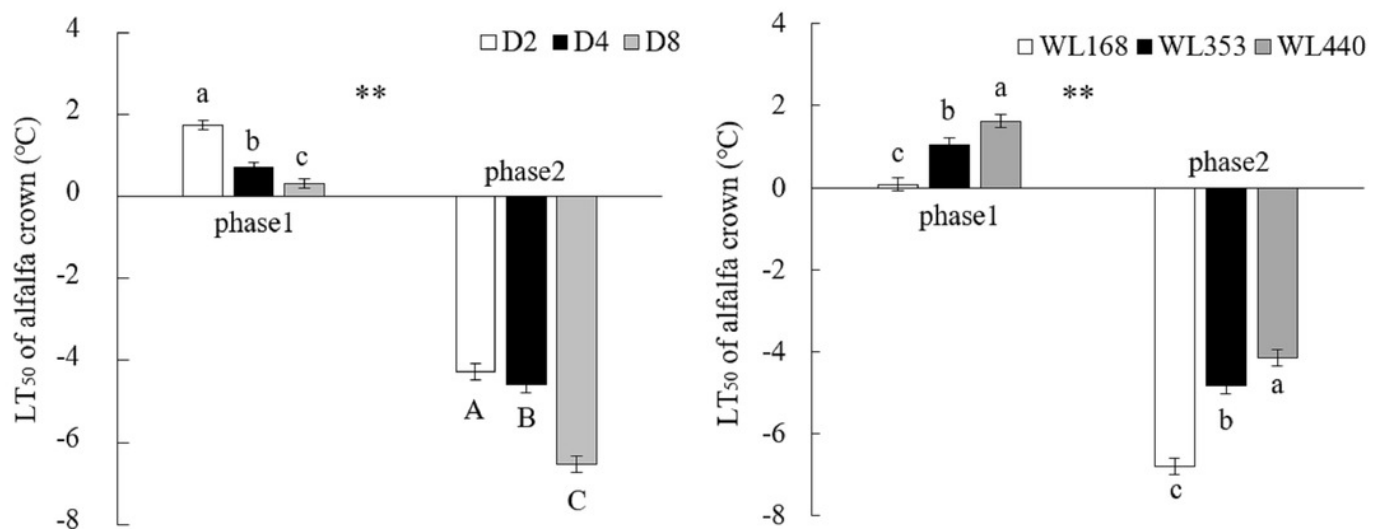
Figure 4

Principal component analysis of 10 variables and the correlations among variables and LT_{50}.

Each arrow represents the eigenvector corresponding to an individual variable. Different colors denote different SRE and shapes refer to cultivars. AGB, above-ground biomass; BGB, below-ground biomass; PRL, primary root length; LRL, lateral root length; CD, crown diameter; RSA, root surface area; RV, root volume; RF, root forks; TI, topological index; FD, fractal dimensions.

□ WL168   ━━━━━ 95% Confidence Ellipse for D$_8$
△ WL353   ━━━━━ 95% Confidence Ellipse for D$_4$
○ WL440   ━━━━━ 95% Confidence Ellipse for D$_2$
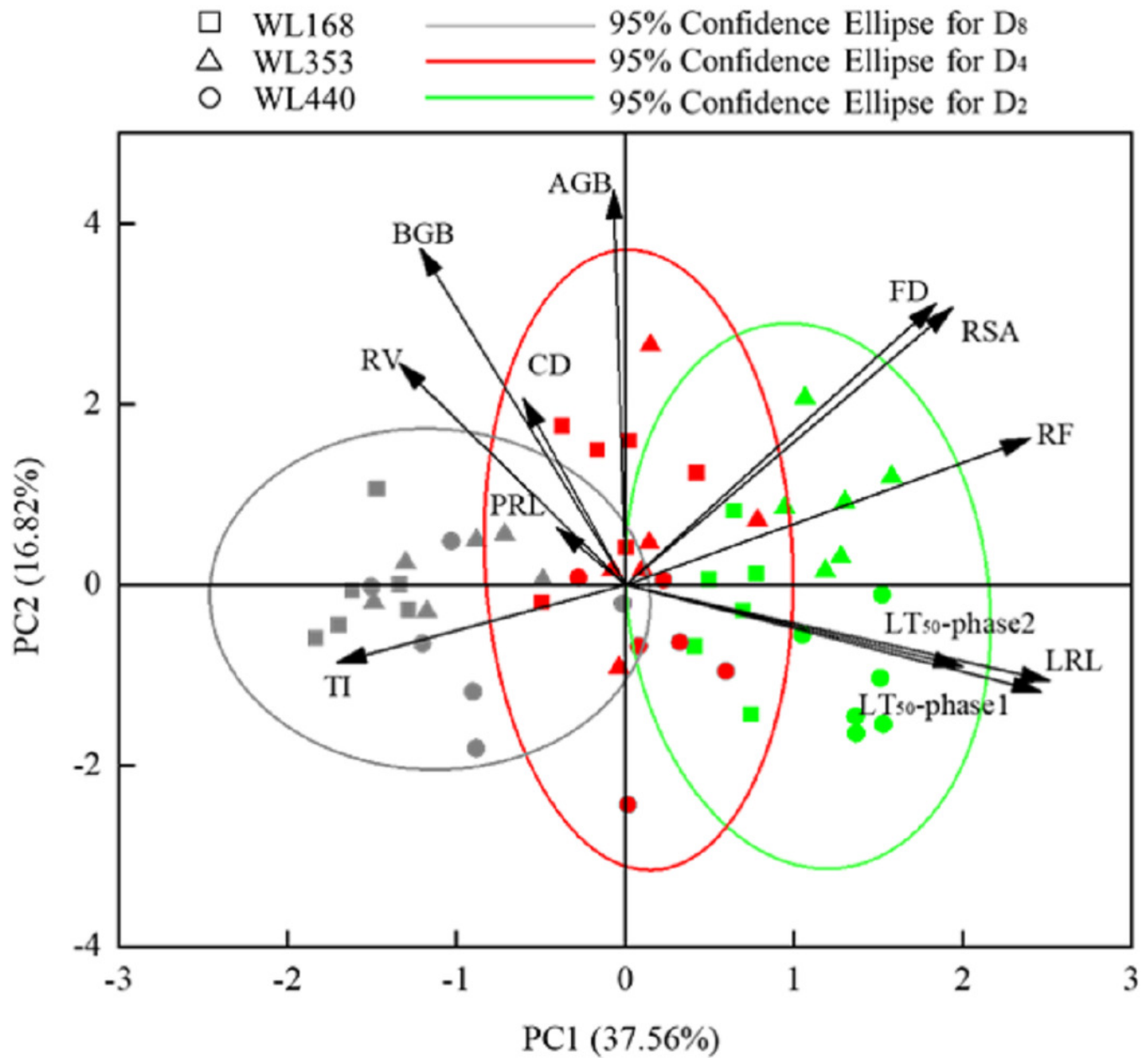
Figure 5

Malondialdehyde content under various simulated rainfall events for the three cultivars in the two phases.

Mean values (n = 18) ± standard errors of the mean are shown. Different capital letters and lowercase letters indicate a significant difference between three simulated rainfall events and cultivars at the same phase at 0.01 and 0.05 level respectively.
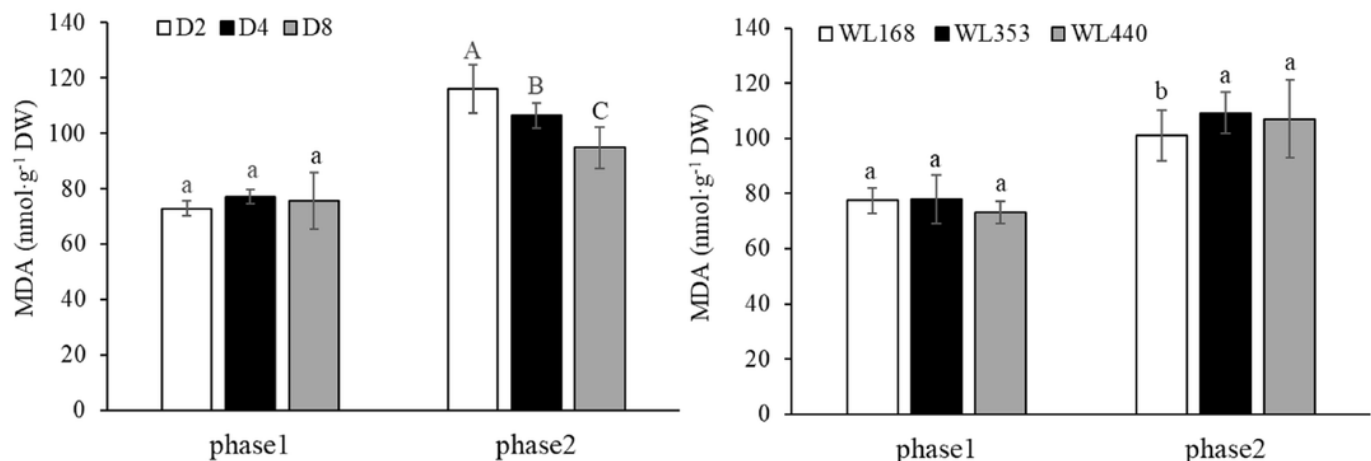
Figure 6

Proline content under various simulated rainfall events for the three cultivars in the two phases.

Mean values (n = 18) ± standard errors of the mean are shown. Different capital letters indicate a significant difference between three simulated rainfall events at the same phase at 0.01 level.
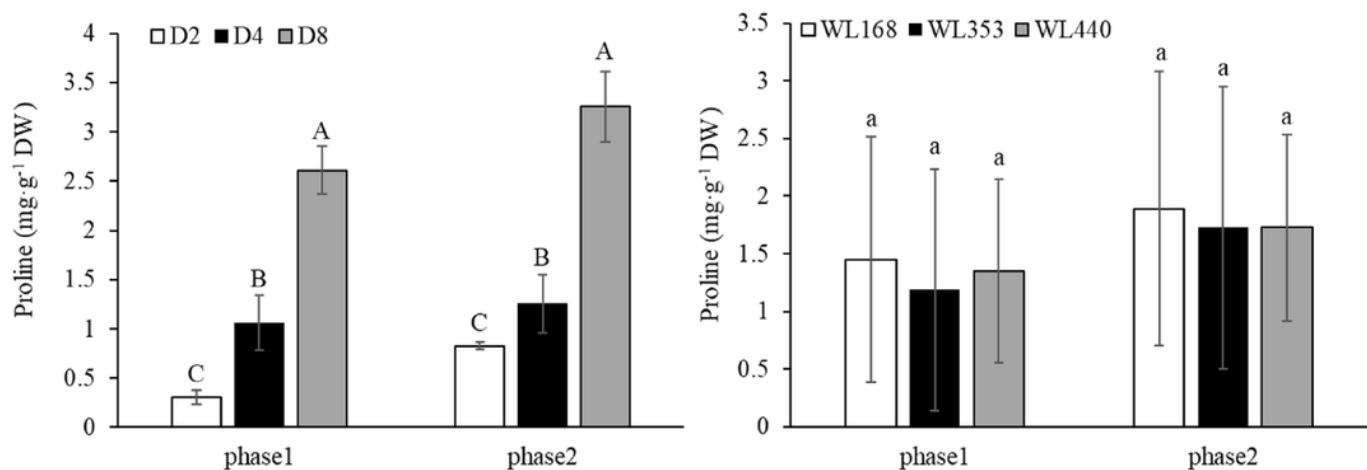
Figure 7

Soluble sugar content under various simulated rainfall events and for the three cultivars.

Mean values (n = 18) ± standard errors of the mean are shown. Different capital letters indicate a significant difference between the three rainfall events at the same phase at 0.01 level.
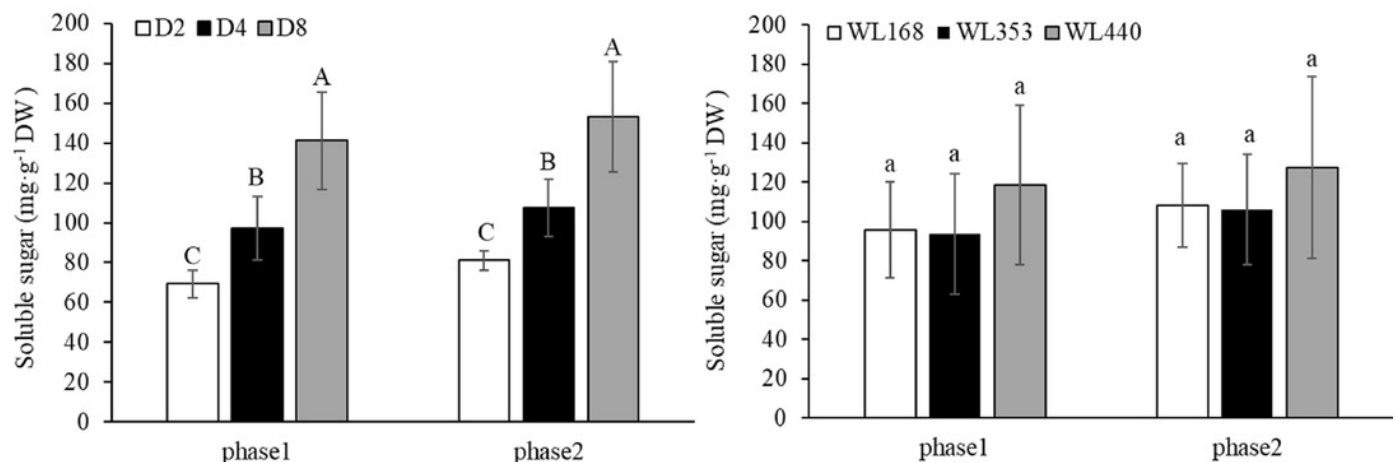
Figure 8

Starch content under various simulated rainfall events for the three cultivars.

Mean values (n = 18) ± standard errors of the mean are shown. Different lowercase letters indicate a significant difference between the three rainfall events at the same phase at 0.05 level.