Evaluation of waterlogging tolerance and responses of protective enzymes to waterlogging stress in pumpkin

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Waterlogging caused by short and severe, or prolonged precipitation can be attributed to global warming. Pumpkin plants are drought-tolerant but not tolerate to waterlogging stress. Under frequent rain and waterlogging conditions, the production of pumpkins is of lower quality, sometimes rotten, and harvest failure occurs in severe cases. Therefore, it is of great significance to assess the waterlogging tolerance mechanism of pumpkin plants. In this study, 10 novel pumpkin varieties from Baimi series were used. The waterlogging tolerance level of pumpkin plants was evaluated by measuring waterlogging tolerance coefficient of biomass and physiological indices using waterlogging stress simulation method. The criteria to evaluate the waterlogging tolerance capacities of pumpkin plants were also explored. Using principal component and membership function analysis, waterlogging tolerance levels of the pumpkin varieties were ranked as follows: Baimi No. 10> Baimi No. 5> Baimi No. 1> Baimi No. 2> Baimi No. 3> Baimi No. 7> Baimi No. 9> Baimi No. 6> Baimi No. 4> Baimi No. 8. Based on the results, Baimi No. 10 was identified with strong waterlogging tolerance and Baimi No. 8 with weak waterlogging tolerance. The responses of malondialdehyde (MDA), proline, key enzymes responsible for anaerobic respiration, and antioxidant enzymes to waterlogging stress were studied in pumpkin plants. The relative expression levels of related genes were determined using real-time fluorescence quantitative PCR technique. The aim of our study was to assess the waterlogging tolerance mechanism of pumpkin plants, thus laying a theoretical foundation for breeding waterlogging-tolerant varieties in the future. After flooding stress treatment, the antioxidant enzyme activities, contents of proline and alcohol dehydrogenases of Baimi No. 10 and Baimi No. 8 displayed an increase followed by a decrease. All indices of Baimi No. 10 were higher than Baimi No. 8. MDA contents gradually increased, with the content being higher in Baimi No. 8 than Baimi No. 10. The activities of pyruvate decarboxylases (PDCs) in Baimi No. 8 and Baimi No. 10 exhibited a decrease initially, followed by an increase, and then a decrease again. The PDC activity in Baimi No. 8 was generally higher

than Baimi No. 10. The relative expression levels of genes encoding superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase were consistent with their corresponding enzyme activities. During the early stage of flooding stress, pumpkin plants waterlogging tolerance was improved by enhancing the expression levels of antioxidant enzyme encoding genes and increasing the antioxidant enzyme activities.

1 **Evaluation of Waterlogging Tolerance and Responses of Protective Enzymes**

2 **to Waterlogging Stress in Pumpkin**

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Abstract:

Waterlogging caused by short and severe, or prolonged precipitation can be attributed to global warming. Pumpkin plants are drought-tolerant but not tolerate to waterlogging stress. Under frequent rain and waterlogging conditions, the production of pumpkins is of lower quality, sometimes rotten, and harvest failure occurs in severe cases. Therefore, it is of great significance to assess the waterlogging tolerance mechanism of pumpkin plants. In this study, 10 novel pumpkin varieties from Baimi series were used. The waterlogging tolerance level of pumpkin plants was evaluated by measuring waterlogging tolerance coefficient of biomass and physiological indices using waterlogging stress simulation method. The criteria to evaluate the waterlogging tolerance capacities of pumpkin plants were also explored. Using the principal component and membership function analysis, waterlogging tolerance levels of the pumpkin varieties were ranked as follows: Baimi No. 10> Baimi No. 5> Baimi No. 1> Baimi No. 2> Baimi No. 3> Baimi No. 7> Baimi No. 9> Baimi No. 6> Baimi No. 4> Baimi No. 8. Based on the results, Baimi No. 10 was identified with strong waterlogging tolerance and Baimi No. 8 with weak waterlogging tolerance. The levels of malondialdehyde (MDA), proline, key enzymes responsible for anaerobic respiration, and antioxidant enzymes to waterlogging stress were studied in pumpkin plants. The relative expression levels of related genes were determined using real-time fluorescence quantitative PCR technique. Our study aimed to assess the waterlogging tolerance mechanism of pumpkin plants, thus laying a theoretical foundation for breeding waterlogging-tolerant varieties in the future. After flooding stress treatment, the antioxidant enzyme activities, contents of proline and alcohol dehydrogenases of Baimi No. 10 and Baimi No. 8 displayed an increase followed by a decrease. All indices of Baimi No. 10 were higher than Baimi No. 8. MDA contents gradually increased, with the content being higher in Baimi No. 8 than Baimi No. 10. The activities of pyruvate decarboxylases (PDCs) in Baimi No. 8 and Baimi No. 10 exhibited a decrease initially, followed by an increase, and then a decrease again. The PDC activity in Baimi No. 8 was generally higher than Baimi No. 10. The relative expression levels of genes encoding superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase were consistent with their corresponding enzyme activities. During the early stage of flooding stress, pumpkin plants waterlogging tolerance was improved by enhancing the expression levels of antioxidant enzyme encoding genes and increasing the antioxidant enzyme activities.

Keywords: Anaerobic respiration key enzymes, Antioxidant enzymes, Change trend, Relative gene expression, RT-qPCR, Waterlogging tolerance evaluation

1 Introduction

In recent years, waterlogging has increased significantly around the globe due to the EI Niño phenomenon (Tang L et al. 1998). Under waterlogging stress, the normal growth of crops is hindered, resulting in reduced yield or even harvest failure in severe cases(Tian L 2019). Pumpkins(*Cucurbita moschata (Duch. ex Lam.) Duch.*) are drought-tolerant crops that are very sensitive to flooding. Frequent rain and waterlogging result in the deterioration of pumpkin quality, rotten melons, and loss of harvest. It not only affects the quality and yield of pumpkins, but also seriously impacts agricultural production and farmers' income. Waterlogging tolerance of crops is a complex quantitative trait that is influenced by multiple factors and the mechanisms also vary

77 with different crops. After flooding, various morphological, physiological, and biochemical indices of crops

78 can be used as an evaluation indicator to measure their waterlogging tolerance(Shi M et al.2006). Based on

79 the phenotypic changes of chrysanthemum during flooding, Yin D et al.(2009) established an evaluation

80 system to measure waterlogging resistance for *chrysanthemum* using the appearance and morphological

81 indicators (e.g., leaf color). At present, research on the waterlogging tolerance of vegetables mostly focuses

82 only on a few crops, such as eggplant, cucumber, and pepper, and there are few research reports regarding the

83 waterlogging tolerance of pumpkins. In this study, we used the multi-factor membership function to evaluate

84 the waterlogging tolerance of pumpkin and conducted principal component analysis (PCA) to enhance the

85 reliability of our evaluation method (Yang X et al.2016;Qi X et al.2011;Zheng J et al.2015;Li T 2007).

86 Under normal growth conditions, the production and scavenging of reactive oxygen species (ROS) in crops

87 cell are in a dynamic equilibrium equilibrium state, this balance is disrupted when crops encounter stress

88 stimuli,it leads to ROS production and metabolism disorders in crops. The antioxidant enzyme system is an

89 important system for scavenging ROS in plants, and it can resist the toxic by-products produced from stress-

90 induced metabolism. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbic acid

91 peroxidase (APX) are key enzymes in the antioxidant system (Imahori Y et al.2008; Miller G et al.2010). The

92 APX activity of Solanaceae plants increases significantly under waterlogging stress, which plays a key role in

93 the scavenging mechanism of H_2O_2 (Lin K 2004). After the watermelon seedlings were induced with

94 waterlogging stress in the leaves, a sequence of events occurred, including the inhibition of SOD activity, the

95 increase of ROS levels, and an increase in SOD and POD activities to scavenge ROS. With the increase in

96 stress, ROS levels further increased, causing membrane lipid peroxidation or delipidation of membrane lipids,

97 which destroyed the membrane structure. Such high ROS levels also led to the accumulation of

98 malondialdehyde (MDA), caused a decrease in the activity of protective enzymes, and damaged the plasma

99 membrane (Liu W *et al*.2016). After stress treatment, the MDA content of peony leaves increased significantly,

100 and a greater increase was observed with a stronger stress level and longer treatment time (Wang J 2015). The

101 MDA content of bitter gourd increased significantly after 4 days of flooding, indicating that the degree of

102 waterlogging was directly proportional to the accumulation of MDA (Zhu J et al.2016). Under waterlogging

103 stress, osmotic regulators have certain effects on ROS scavenging. For example, the increase in proline content

104 is associated with the effective scavenging of ROS (Jiang M 1999). After 10 days of flooding, the proline

105 content in cucumber was significantly higher than that of the control, with an increase of 58.9% (Barickman

106 TC et al.2019).

107 Soil hypoxia is caused by waterlogging stress, which inhibits the aerobic respiration pathway of roots,

108 increases the activity of anaerobic respiration enzymes, and strengthens the anaerobic respiration pathway.

109 Alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) are key enzymes in the anaerobic

110 respiration pathways (Baileyserres J et al.2005). ADH-deficient transgenic plants were more sensitive to

111 waterlogging, indicating that ethanol fermentation plays an important role in the plants mechanism of

112 waterlogging tolerance (Thomas AL et al.2005). Additionally, the activities of ADH and PDC were accelerated,

113 and the concentrations of ethanol, acetaldehyde, and lactic acid were increased after the cucumber was

114 submerged in water for 48 hours(Xu X et al.2014).

115 Different crops exhibit distinct resistance levels during waterlogging stress, and the expression of related genes

116 is greatly associated with genotype. Under adverse stress conditions, some related genes are either induced or

117 silenced, and their products resist the environmental stresses causing morphological and physiological changes

118   in plants that affect their normal growth (Zhang Y et al.2011;Long C 2019). Since real-time fluorescence
119   quantitative polymerase chain raction (RT-qPCR) was invented, it has widely been used in various fields and is
120   one of the important techniques to quantitatively detect gene expression (Wang S et al.2013; Tian P et al.2015).
121   Xu used RT-qPCR technology to measure the relative expression of waterlogging tolerance-related genes in
122   tea plants (Xu Y 2016).
123   The characteristics of different varieties of the same crop vary greatly, including waterlogging tolerance level.
124   Different stress levels and stress durations have different effects on crops. Therefore, it is of great significance to
125   study the waterlogging tolerance of different varieties of pumpkin. At present, there is no recognized standard
126   to evaluate the waterlogging tolerance of melons. In order to meet the needs of market structure and improve the
127   competitiveness of Chinese pumpkin, research group has selected and bred a new pumpkin variety of Baimi
128   series with strong growth potential and excellent quality in the early more than ten years of work. The mother and
129   father are both obtained by self-crossing and purification of single plant of high generation. In this study,
130   pumpkin varieties of the Baimi seriesIn this study, pumpkin varieties of the Baimi series were used, and biomass
131   and physiological indices were determined using waterlogging stress simulation method. PCA and
132   membership function analyses were used to identify waterlogging-tolerant varieties of pumpkin and an
133   evaluation method was devised to assess their waterlogging tolerance. Waterlogging simulation was used to
134   measure the stress response of identified varieties with strong or weak waterlogging tolerance, including changes
135   in MDA content, proline content, and activities of key enzymes for anaerobic respiration and antioxidant
136   enzymes under waterlogging stress. The relative expression levels of related genes were determined using RT-
137   qPCR technique.The response of pumpkin with different waterlogging tolerance to waterlogging stress was
138   studied.This study aimed to explore the mechanisms of waterlogging tolerance in pumpkins, thus laying a
139   theoretical foundation for breeding novel waterlogging-resistant pumpkin varieties..

140   **2 Materials and methods**

141   **2.1 Experimental materials**

142   The experiment was conducted on 10 pumpkin varieties, including Baimi No. 1, Baimi No. 2, Baimi No. 3,
143   Baimi No. 4, Baimi No. 5, Baimi No. 6, Baimi No. 7, Baimi No. 8, Baimi No. 9, and Baimi No.10. All
144   varieties were provided by the Henan Institute of Science and Technology, Henan, China.

145   **2.2 Experimental design**

146   Waterlogging stress simulations were carried out in the laboratory of the School of Horticulture and Landscape
147   Architecture, Henan University of Science and Technology, from June to July and October to December, 2021.
148   The experiment consisted of two treatments—under conventional (control) and flooded conditions, with a
149   randomized block design and three replicates for each treatment.

150   A total of 50 seeds (fully developed, no pests, and of similar shape) of each of the 10 tested varieties were
151   sown in nutrient pots after germination through soaking.The seeds were sown in a nutritive pot with a special
152   seedling substrate,Light, temperature and humidity were under conventional management. When the seedlings
153   developed two full leaves and one terminal shoot, the double-pot method (Liu C et al.2020) was used under
154   flood stress treatment. The water surface was 2-3 cm higher than the substrate, and plants were watered every
155   day. The control group was managed using standard practices applied in the area. On the 7th day of stress
156   treatment under flooding, leaves and root were collected to measure biomass, relative chlorophyll content,
157   antioxidant enzyme activity, and MDA content.

158   Baimi No. 10 (strong waterlogging tolerance) and Baimi No. 8 (weak waterlogging tolerance) varieties were

159 selected for the further experiment, and the treatment method remained the same as above. Leaves and roots
160 were taken at 0 (control),1,3,5, and 7d after the treatment. Antioxidant enzyme activity, MDA and proline
161 content, activities of key enzymes responsible for anaerobic respiration and antioxidant enzymes were
162 determined. The relative expression of related genes was determined using real-time fluorescence quantitative
163 PCR technique.

164 **2.3 Measurement methods**

165 The relative content of chlorophyll was determined using a portable chlorophyll instrument, soil and plant
166 analyzer development (SPAD)-502. The SOD and POD activities were measured by the nitrogen blue
167 tetrazolium photoreduction and guaiacol methods, respectively. MDA content was determined by the
168 thiobarbituric acid method. Proline (PRO) content was determined by acid ninhydrin method.Ultraviolet (UV)
169 spectrophotometry was used to measure CAT, APX, ADH, and PDC activities (Li H 2000). The detection kits
170 to measure all these indices were purchased from Beijing Solarbio Technology Co., Ltd.Take SOD for
171 example:0.1g pumpkin leaves were weighed and 1mL extract was added to make ice bath homogenate.
172 Centrifuged at 4℃ for 10min,8000 rpm, then corresponding reagents were added in turn according to the
173 requirements of the kit.Thoroughly mixed and placed in 37℃ water bath for 30min.The absorption value A was
174 measured at 560nm.Percentage inhibition=[(A1$_{blank}$-A2$_{blank}$)-(A$_{measure}$-A$_{contrast}$)]÷(A1$_{blank}$-A2$_{blank}$)×100%. SOD
175 activity(U/g)=[percentage inhibition÷(1-percentage inhibition)×V$_{RT}$]÷(W×V$_{S}$÷V$_{ET}$)×F. V$_{RT}$:Total volume of
176 reaction,0.2mL;V$_{S}$:sample volume,0.02mL;V$_{ET}$:Total volume of extract; W:sample weight,g;F:Sample dilution
177 ratio.

178 RNA extraction and cDNA synthesis: Total RNA was extracted from plant leaves following the
179 instructions(Takara Bio Inc. MiniBEST Plant RNA Extraction Kit) mentioned in the RNA extraction kit.
180 NanoDrop 2000 was used and the absorption value A was measured at 260nm to detect the concentration and
181 purity of the total RNA. Qualified and quantified total RNA was reverse transcribed into cDNA using Takara
182 Bio Inc.PrimeScript™ II 1st Strand cDNA Synthesis Kit.Reverse transcription reaction system 1 plus RNase free
183 dH2O to 10 μl was mixed and incubated at 65 ℃ for 5 min,then ice bath quickly. The mixture of reverse
184 transcription reaction system 2 was added into the test tube of ice bath, heated at 42 ℃ for 30-60 min, heated at
185 95 ℃ for 5 min to end the reaction, and put on ice for subsequent experiments or cryopreserved.(Table 1)

186 RT-qPCR: The primer sequences of genes encoding SOD, POD, CAT, and APX were designed according to
187 Zheng et al (Zheng J 2020), and β-action was used as the housekeeping gene (Table 2).According to the qPCR
188 reaction system(Table 3), they were added successively, mixed and centrifuged. The reaction solution was placed
189 on the Realtime PCR instrument and PCR was carried out using the following parameters: 95 ℃ for 5 min, 95 ℃
190 for 15 s, 60 ℃ for 30 s, and a total of 40 cycles. After the reaction, the amplification and melting curves were
191 observed, and the data were analyzed using the $2^{-\Delta\Delta CT}$(Radio(test/calibrator)=$2^{-[\Delta CT(test)-\Delta CT(calibrator)]}$; ΔCt=Ct
192 target gene-Ct reference gene; $\Delta\Delta$Ct=ΔCt test-ΔCt contrast; Fold Change:$2^{-\Delta\Delta Ct}$)method (Kenneth JL et
193 al.2001). The relative expressions of the enzyme genes were calculated using the above-mentioned method.

194 **2.4 Statistical analysis**

195 Waterlogging tolerance coefficient (WTC) was calculated using Equation 1 as follows:

196 WTC=Measured value (treatment)∕Measured value (control) (1)

197 Based on the calculated WTC values, PCA was performed to obtain comprehensive indices.

198 The membership function (MF) was calculated using Equation 2 as follows:

199

200
$$\mathrm{MF}(X_j) = (X_j - X_{min})/(X_{max} - X_{min}) \; (2)$$

201 where, j=1, 2, ... n; X_j represents the jth comprehensive index; X_{min} represents the minimum value of the jth
202 comprehensive index; and X_{max} represents the maximum value of the jth comprehensive index.

203 Weightness (W) was calculated according to the contribution rate of each principal component using Equation

204 3: $W_j = P_j = \sum_{j=1}^{n} P_j \# (3) = P_j / \sum_{j=1}^{n} P_j \; (3)$

205 where, j=1, 2, ... n; W_j represents the importance of the jth comprehensive index among all the comprehensive
206 indices, and P_j is the contribution rate of the jth comprehensive index of each variety.

207 Comprehensive evaluation value (CEV) to measure waterlogging tolerance was calculated using Equation 4:

208 $\mathrm{CEV} = \sum_{j=1}^{n} \left[U(X_j) \times W_j \right] \; (4)$

209 where, j = 1, 2, ..., n. D is the comprehensive evaluation value of waterlogging tolerance obtained from the
210 comprehensive indices of one tested variety under waterlogging stress conditions.
211 Statistical analysis of data was performed using DPS 7.55 and SPSS 21.0. Duncan's new multiple range
212 method was used for variance analysis, and GraphPad Prism 8.0 was used for plotting ($P<0.05$).
213 **3 Results**
214 **3.1 Evaluation of waterlogging tolerance of Baimi pumpkin varieties**
215 The WTC of each index was calculated using Equation 1. The results of our study show that the WTC values
216 of fresh aboveground mass were the largest for Baimi No. 2. The WTC values of underground dry mass and
217 fresh mass were the largest for Baimi No. 3. The WTC values of aboveground dry mass were the largest for
218 Baimi No. 5. The WTC values for SOD, POD, and CAT activities were the largest for Baimi No. 10. The
219 WTC of MDA content was the largest for Baimi No. 1. The WTC of the Chlorophyll SPAD value was the
220 largest for Baimi No. 6 (Table 4).
221 Principal component analysis(PCA): PCA was carried out based on the WTCs of 9 indices of pumpkin
222 seedlings. The contribution rates of the first three principal components were 46.381%, 28.418%, and 12.032%.
223 The cumulative contribution rate was 86.831%, indicating that the first three principal components explained
224 86.831% of the total variations.
225 Membership function analysis: The membership function value of waterlogging tolerance indices of each
226 pumpkin variety was calculated using Equation 2. For the first principal component, Baimi No. 10 had the
227 largest $MF(X_1)$, and displayed the highest degree of waterlogging tolerance. It can be seen from PC_1 that
228 indices with a large coefficient value showed a positive correlation with waterlogging tolerance. For the
229 second principal component, Baimi No. 1 had the largest $MF(X_2)$ and displayed the highest degree of
230 waterlogging tolerance. The value of Baimi No. 8 was the smallest, and thus the least waterlogging-tolerant
231 variety. It can be seen from PC_2 that MDA contents with a large coefficient value had a negative correlation
232 with waterlogging tolerance. Based on this relationship, Baimi No. 1 was the most resistant to waterlogging,
233 which is a false interpretation. For the third principal component, Baimi No. 10 had the largest $MF(X_3)$, and
234 displayed the highest degree of tolerance to waterlogging. The values for Baimi No. 3 were the smallest,
235 indicating it to be the least resistant to waterlogging (Table 5).
236 Comprehensive evaluation: The comprehensive evaluation value (CEV) was calculated using Equation 4.
237 Based on the CEV values, 10 pumpkin varieties were sorted according to their waterlogging tolerance levels as

238 follows: Baimi No. 10> Baimi No. 5> Baimi No. 1> Baimi No. 2>Baimi No. 3>Baimi No. 7>Baimi No.

239 9>Baimi No. 6>Baimi No. 4>Baimi No. 8 (Table 5).

240 **3.2 Response of pumpkin varieties with different waterlogging tolerance to waterlogging stress**

241 **3.2.1 Response of antioxidant enzymes to waterlogging stress in pumpkin**

242 During waterlogging stress, the activities of SOD, POD, CAT, and APX in Baimi No. 8 and Baimi No. 10

243 showed first an increasing trend followed by a decrease. The activities of SOD, CAT, and APX for Baimi No.

244 10 were higher than those of Baimi No. 8, whereas the POD activity of Baimi No. 8 was higher than Baimi No.

245 10. The activities of SOD, POD, and CAT reached their highest level on the 3rd day, 1.08 times and 1.29 times,

246 2.24 times and 2.32 times, 3.04 times and 4.19 times higher than the control group, respectively. On the 7th

247 day, the SOD activities of Baimi No. 8 and Baimi No. 10 increased by 8.26% and 29.1%, respectively, as

248 compared to the control group. The POD activity decreased by 0.98 times and 1.16 times as compared to the

249 control values. The CAT activity of Baimi No. 8 decreased by 23.93% as compared to the control, while the

250 CAT activity of Baimi No. 10 increased by 28.30% as compared to the control. The APX activities of these

251 two varieties were always lower than the control and were observed to be lowest on the 7th day. The

252 antioxidant enzyme activities increased during the early stages of waterlogging stress (Figures 1-4).

253 **3.2.2 Response of MDA and proline contents to waterlogging stress in pumpkin**

254 The MDA content in the leaves of the two pumpkin varieties gradually increased when the waterlogging stress

255 was prolonged. The MDA contents of Baimi No. 8 and Baimi No. 10 were the highest on the 7th day, being

256 2.09 and 1.62 times higher than the control, respectively. The MDA content of Baimi No. 8 was always

257 observed to be higher than Baimi No. 10, indicating that Baimi No. 8 had a higher degree of membrane lipid

258 peroxidation, and the damage was greater under waterlogging stress (Figure 5).

259 During the waterlogging stress, the proline contents of Baimi No. 8 and Baimi No. 10 first displayed an

260 increase followed by a decrease, and reached their peak on the 3rd day, being 1.60 and 4.92 times higher than

261 the control, respectively. During the first phase, the increase in proline content of Baimi No. 10 was 1.49 times

262 higher than the control followed by a phase that exhibited a decrease in which the contents of Baimi No. 10

263 were 0.61 times higher than the control (Figure 6).

264 **3.2.3 Response of key enzymes responsible for anaerobic respiration to waterlogging stress in pumpkin**

265 **root**

266 The two cultivars first exhibited an increase in their ADH activities followed by a decrease when the

267 waterlogging stress was prolonged. The ADH activity of Baimi No. 10's was higher than Baimi No. 8, which

268 is always lower than the control values. The ADH activity of Baimi No. 10 was observed to be the highest on

269 the 5th day. On the 7th day, the ADH activities of Baimi No. 8 and Baimi No. 10 decreased by 73.33% and 25%

270 than the control, respectively. The PDC activities of Baimi No. 8 and Baimi No. 10 showed a trend that

271 decreased first, rose and then decreased again. The PDC activity of Baimi No. 10's was generally higher than

272 the Baimi No. 8. Baimi No. 8 was always lower than the control values. On the 1st day, the PDC activity of

273 Baimi No. 10 increased 72.09% higher as compared to the control. The PDC activities of the Baimi No. 8 and

274 Baimi No. 10 decreased to their lowest value on the 3rd day, with 58.50% and 56.28% lower than the control,

275 respectively. The activities of key enzymes responsible for anaerobic respiration were higher in the variety

276 with strong waterlogging tolerance as compared to the one with lower tolerance (Figures 7-8).

277 **3.2.4 Response of pumpkin antioxidant enzyme genes expressions to waterlogging stress**

278 During the waterlogging stress, the expression levels of genes encoding SOD, POD, CAT, and APX in Baimi

279 No. 8 and Baimi No. 10 all exhibited an increase first followed by a decrease. The genes encoding SOD, POD,
280 and CAT were highly expressed on the 3rd day, being 3.05 and 18 times, 3.05 and 2.37 times, 8.87 and 11.56
281 times than the control, respectively. The expression level of the gene encoding APX in Baimi No. 10 increased
282 to its highest level on the 3rd day, being 7.78 times higher than the control, and in Baimi No. 8 it was the
283 highest on the 5th day. The relative expression levels of genes encoding SOD, CAT, and APX in Baimi No. 10
284 were higher than the Baimi No. 8, and the relative expression levels of the gene encoding POD in Baimi No. 8
285 were higher than the Baimi No. 10. The trends of antioxidant enzyme gene expression levels were consistent
286 with their corresponding enzyme activities (Figures 9-12).

287 **4 Discussion**

288 In previous studies, the indices to measure waterlogging tolerance have been screened out in vegetables and
289 mainly focused on growth, physiological, and biochemical indicators. In this study, 10 pumpkin varieties were
290 used to assess the waterlogging tolerance level of pumpkins. PCA and membership function analysis were
291 used to convert 9 indices to 3 independent comprehensive indicators, including biomass, relative chlorophyll
292 content, antioxidant enzyme activity, and MDA content. The corresponding membership function values were
293 weighted to obtain the comprehensive evaluation value (CEV)of waterlogging tolerance. Gao et al.(2018)
294 screened out fresh underground mass, SOD, and MDA content as valid indicators for rapid evaluation of
295 waterlogging tolerance in broccoli seedlings , which suggests that growth indices and antioxidant enzyme
296 activities can be used as an indicator to evaluate waterlogging tolerance.

297 During the initial stage of waterlogging stress, ROS accumulation in plants was accompanied by improved
298 antioxidant enzyme activities such as SOD, POD, CAT, and APX, protecting plants from ROS damage
299 (Limón-Pacheco J et al.2008).This was an important pathway that helps plants cope with all kinds of stress.SOD
300 was considered as first defense against ROS as it acted on superoxide radicals, which were produced in different
301 compartments of the cell and acted as precursor to other ROS(Alscher et al.2002).APX rapidly reduced H_2O_2 to
302 H_2O and O_2 through the ascorbate-glutathione cycle(S.Mishra et al.2006) Li et al.(2007) reported that SOD,
303 CAT, and POD activities in cucumber leaves increased three days before waterlogging occurred, and then
304 gradually decreased . Yang et al.(2014) found that SOD, POD, and CAT activities in tomato leaves first
305 exhibited an increase followed by a decrease under water stress conditions . In this study, SOD, POD, CAT,
306 and APX activities in pumpkin also displayed an increase first and then a decrease under waterlogging stress,
307 which is consistent with Li et al.(2007) and Yang et al.(2014), indicating that short-term waterlogging stress
308 stimulated the antioxidant system of plants, formed a certain waterlogging resistance mechanism, and maintained
309 a high protective enzyme.However, long-term stress would cause certain damage to the antioxidant system, so
310 that its antioxidant capacity gradually faded. The antioxidant enzyme activity of Baimi No. 10 was higher than
311 the Baimi No. 8, indicating that Baimi No. 10 has an efficient enzymatic scavenging system to regulate the
312 ROS levels. The SOD, POD, CAT, and APX activities in waterlogging-tolerant maize have been reported to
313 enhance to a larger extent as compared to varieties that are sensitive to waterlogging stress(Wang J et al.2018).
314 MDA is an important indicator to measure plant injury under stressed conditions. Zhu et al. found that the
315 higher the MDA content, the weaker the waterlogging resistance in bitter gourd(Zhu J et al.2016). In this study,
316 the MDA content continued to increase when waterlogging stress was prolonged. The MDA content of Baimi
317 No. 8 was higher than Baimi No. 10, and these results were consistent with Zheng et al.(2020), where the
318 MDA content in waterlogging-sensitive watermelon varieties was significantly higher than the waterlogging-
319 resistant varieties. Under stress conditions, the important role of proline may be as a scavenger and penetrant to

320 improve crop growth characteristics and yield(Khaled A. A. Abdelaal et al.2020).Wang et al.(2010) found that
321 after waterlogging, the content of free proline in melons increased first and then decreased. The proline content
322 in waterlogging-tolerant varieties was significantly higher than the sensitive varieties. In this study, under
323 waterlogging stress, the proline contents of Baimi No. 8 and Baimi No. 10 both followed a similar trend with
324 an increase first and then a decrease. The proline content of Baimi No. 10 was higher than Baimi No. 8,
325 indicating that the waterlogging tolerant pumpkin varieties reduced the oxidation of proline to glutamic acid,
326 which made the proline rapid accumulation to reduce the osmotic potential of cells and relieved the damage of
327 waterlogging stress during the early stage(Muneera ALKahtani et al.2021)..
328 Plants respond to waterlogging stress by releasing a small amount of energy for their growth through anaerobic
329 respiration and regulating their metabolic pathways. During anaerobic respiration, ADH is the main enzyme
330 that prolongs the survival time of plants through the Pasteur effect under hypoxic conditions(Chen Y et
331 al.2005). Diao et al. found that under waterlogging stress, ADH and LDH activities increased in melons, and
332 the ADH activity was higher in 'Cuixi', a variety with slightly higher waterlogging tolerance, as compared to
333 'Century Honey', a variety with weaker tolerance (Diao Q et al.2020). In this study, the ADH activities of both
334 Baimi No. 8 and No. 10 displayed and an increasing trend followed by a decrease. The ADH activity in Baimi
335 No. 10 was higher than Baimi No. 8, which is consistent with Diao et al.(2020). The PDC activities of Baimi
336 No. 8 and Baimi No. 10 showed a trend that decreased first, followed by an increase and a decrease again. The
337 PDC activity of Baimi No. 10 was generally higher than Baimi No. 8. These results were consistent with Chen
338 et al.(2007), who reported that the PDC activity of the waterlogging tolerant rootstock variety 'Mahali' was
339 higher than the sensitive variety 'Dongbei Shanying'.
340 Xu (2016) assessed the resistance mechanism of tea plants and found that the relative expressions of SOD
341 genes in leaves increased and those of CAT genes in leaves decreased first and then increased. Chin et
342 al.(2014)found that in loofah plants, the expression of the gene encoding APX was enhanced with the increase
343 in enzyme activity, resulting in an increased scavenging ability of ROS under flooding stress . In this study,
344 under waterlogging stress, the expression levels of SOD, POD, CAT, and APX encoding genes displayed a
345 trend of an increase first and then a decrease, which was consistent with the trend of their corresponding
346 enzyme activities. In Baimi No. 10, the expression of the APX encoding gene was significantly higher than
347 Baimi No. 8, which is consistent with the findings of Xia et al.(2015). The results showed that in the early stage
348 of stress, the expression of antioxidant enzyme genes was enhanced, which significantly increased the enzyme
349 activity. Consequently, the production and scavenging of ROS can maintain an equilibrium state for a certain
350 period, thereby reducing damage to plants.

351 **5 Conclusion**

352   In this study, biomass, antioxidant enzyme activity, and relative chlorophyll content of 10 different
353 pumpkin varieties were used as indicators to evaluate their waterlogging tolerance. Through PCA and
354 membership function analysis, we identified the varieties that were the most tolerant to waterlogging. Among
355 10 tested pumpkin varieties, Baimi No. 10 was the most tolerant and Baimi No. 8 was the least tolerant. Using
356 Baimi No. 10 and Baimi No. 8 varieties, we further studied the responsive activities of MDA, proline, key
357 enzymes responsible for anaerobic respiration, antioxidant enzymes, and the expression of encoding genes to
358 waterlogging stress in pumpkin plants. When Baimi No. 8 was compared with Baimi No. 10, it was found that
359 it may possess a more efficient enzymatic scavenging system to regulate ROS. The MDA content of Baimi No.
360 8 was higher than that of Baimi No.10, and the degree of lipid peroxidation was higher.The relative expression

361 levels of SOD, POD, CAT, and APX encoding genes were consistent with their corresponding enzyme
362 activities.All of them increased first and then decreased, indicating that the expression of antioxidant enzymes in
363 plants was up-regulated by short time of waterlogging stress, and a certain waterlogging tolerance mechanism
364 was formed. However, with the extension of stress time, the antioxidant system was destroyed. In the early stage
365 of waterlogging stress, pumpkin plants may resist waterlogging by enhancing the expressions of antioxidant
366 enzyme-encoding genes and improving enzyme activity. However, after 3 days of stress treatment, the gene
367 expression levels of antioxidant enzymes and their activities decreased. The degree of membrane lipid
368 peroxidation increased, and plant growth was inhibited. The antioxidant enzyme activities and related gene
369 expressions were higher in the variety with strong waterlogging tolerance than in the variety with weak
370 waterlogging tolerance.

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375 experiment.

376 **Supplementary Information**

377 **Certification of English Editing**.

378 **Author contribution statement**

379 Liu Zhenwei and Li Xinzheng designed experiments; Liu Zhenwei and Qiao Dandan and Liu Zhenyu and Yan
380 Xiaowen and Wang Pengwei carried out experiments;Liu Zhenwei and Qiao Dandan and Sun Li analyzed
381 experimental results;Liu Zhenwei and Qiao Dandan and Guo Linxin wrote the manuscript.All authors have
382 read and approved the final manuscript.

383 **Declarations**

384 Conflicts of interest   The authors declare that they have no conflict of interest.

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528 **Tables:**
529 Table 1 Retrotranscriptional response system
530 Table 2 Primer sequences used for real-time fluorescence quantification
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533 under the waterlogging
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535 pumpkin varieties
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537 **Figure legends**
538 Fig 1 Response of superoxide dismutase(SOD) to waterlogging stress in pumpkin
539 leaves.Different letters above columns indicate that the difference of superoxide dismutase
540 activitiesis significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
541
542 Fig 2 Response of peroxidase(POD) to waterlogging stress in pumpkin leaves.Different letters
543 above columns indicate that the difference of peroxidase activities significant under the
544 waterlogging (P < 0.05). Vertical bars = SD (n = 3)
545
546 Fig 3 Response of catalase(CAT) to waterlogging stress in pumpkin leaves.Different letters
547 above columns indicate that the difference of catalase activities significant under the
548 waterlogging (P < 0.05). Vertical bars = SD (n = 3)
549
550 Fig 4 Response of ascorbic acid(APX) to waterlogging stress in pumpkin leaves.Different letters
551 above columns indicate that the difference of ascorbic acid activities significant under the
552 waterlogging (P < 0.05). Vertical bars = SD (n = 3)
553
554 Fig 5 Response of malondialdehyde (MDA) to waterlogging stress in pumpkin leaves.Different
555 letters above columns indicate that the difference of the content of malondialdehyde significant
556 under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
557
558 Fig 6 Response of proline(PRO)to waterlogging stress in pumpkin leaves.Different letters above
559 columns indicate that the difference of the content of proline significant under the waterlogging
560 (P < 0.05). Vertical bars = SD (n = 3)
561
562 Fig 7 Response of alcohol dehydrogenase(ADH) to waterlogging stress in pumpkin
563 roots.Different letters above columns indicate that the difference of alcohol dehydrogenase(ADH)
564 activity significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
565

566   Fig 8 Response of pyruvate decarboxylase(PDC) to waterlogging stress in pumpkin
567   roots.Different letters above columns indicate that the difference of pyruvate decarboxylase
568   activity significant under the waterlogging ($P < 0.05$). Vertical bars = SD ($n = 3$)

569

570   Fig 9 Response of gene expression of superoxide dismutase(SOD) to waterlogging stress in
571   pumpkin leaves.Different letters above columns indicate that the difference of the gene
572   expression of superoxide dismutase significant under the waterlogging ($P < 0.05$). Vertical bars =
573   SD ($n = 3$)

574

575   Fig 10 Response of gene expression of peroxidase(POD) to waterlogging stress in pumpkin
576   leaves.Different letters above columns indicate that the difference of the gene expression of
577   peroxidase significant under the waterlogging ($P < 0.05$). Vertical bars = SD ($n = 3$)

578

579   Fig 11 Response of gene expression of catalase(CAT) to waterlogging stress in pumpkin
580   leaves.Different letters above columns indicate that the difference of the gene expression of
581   catalase significant under the waterlogging ($P < 0.05$). Vertical bars = SD ($n = 3$)

582

583   Fig 12 Response of gene expression of ascorbic acid(APX) to waterlogging stress in pumpkin
584   leaves.Different letters above columns indicate that the difference of the gene expression of
585   ascorbic acid significant under the waterlogging ($P < 0.05$). Vertical bars = SD ($n = 3$)

586

587

Figure 1

Response of superoxide dismutase(SOD) to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of superoxide dismutase activitiesis significant under the water logging (P < 0.05). Vertical bars = SD (n = 3)
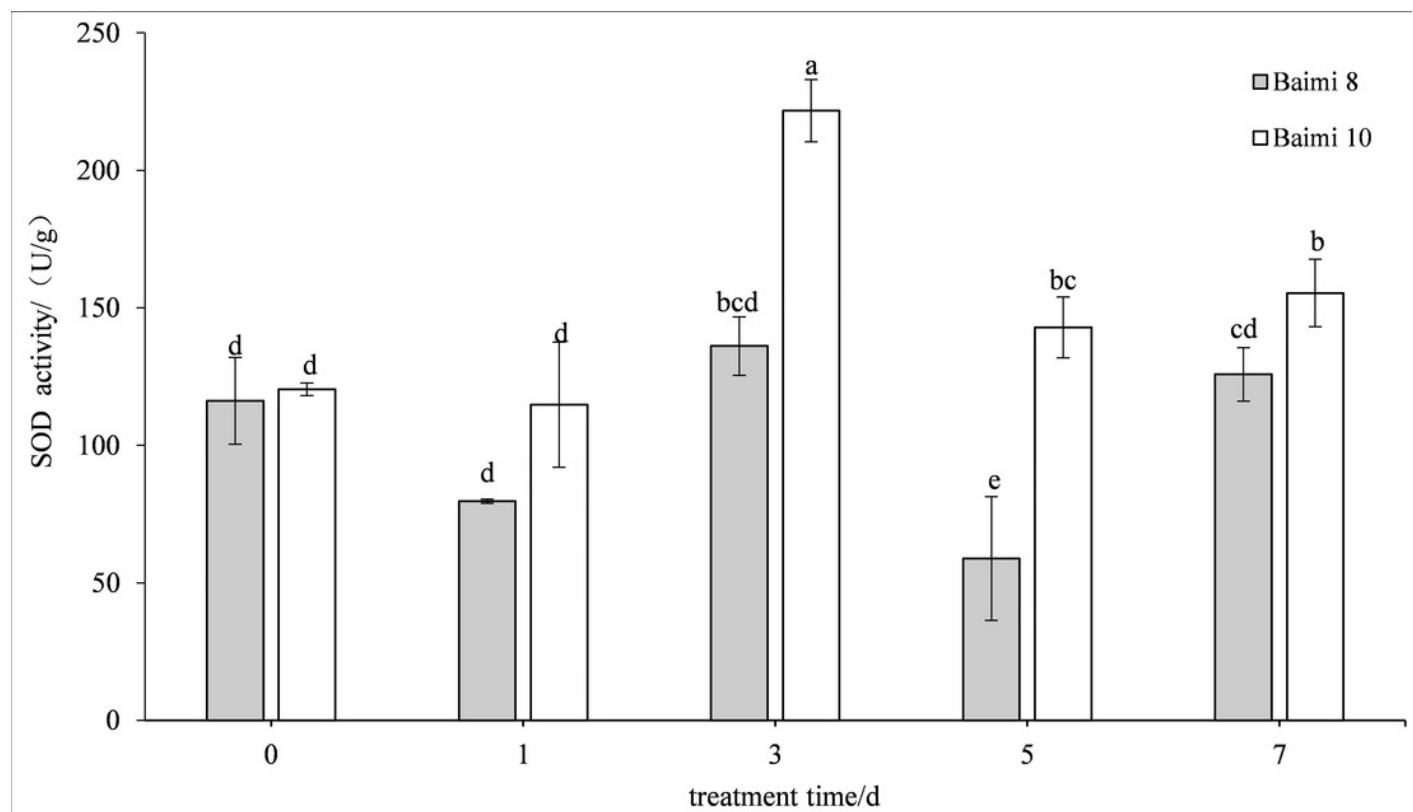
Figure 2

Response of peroxidase(POD) to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of peroxidase activities significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
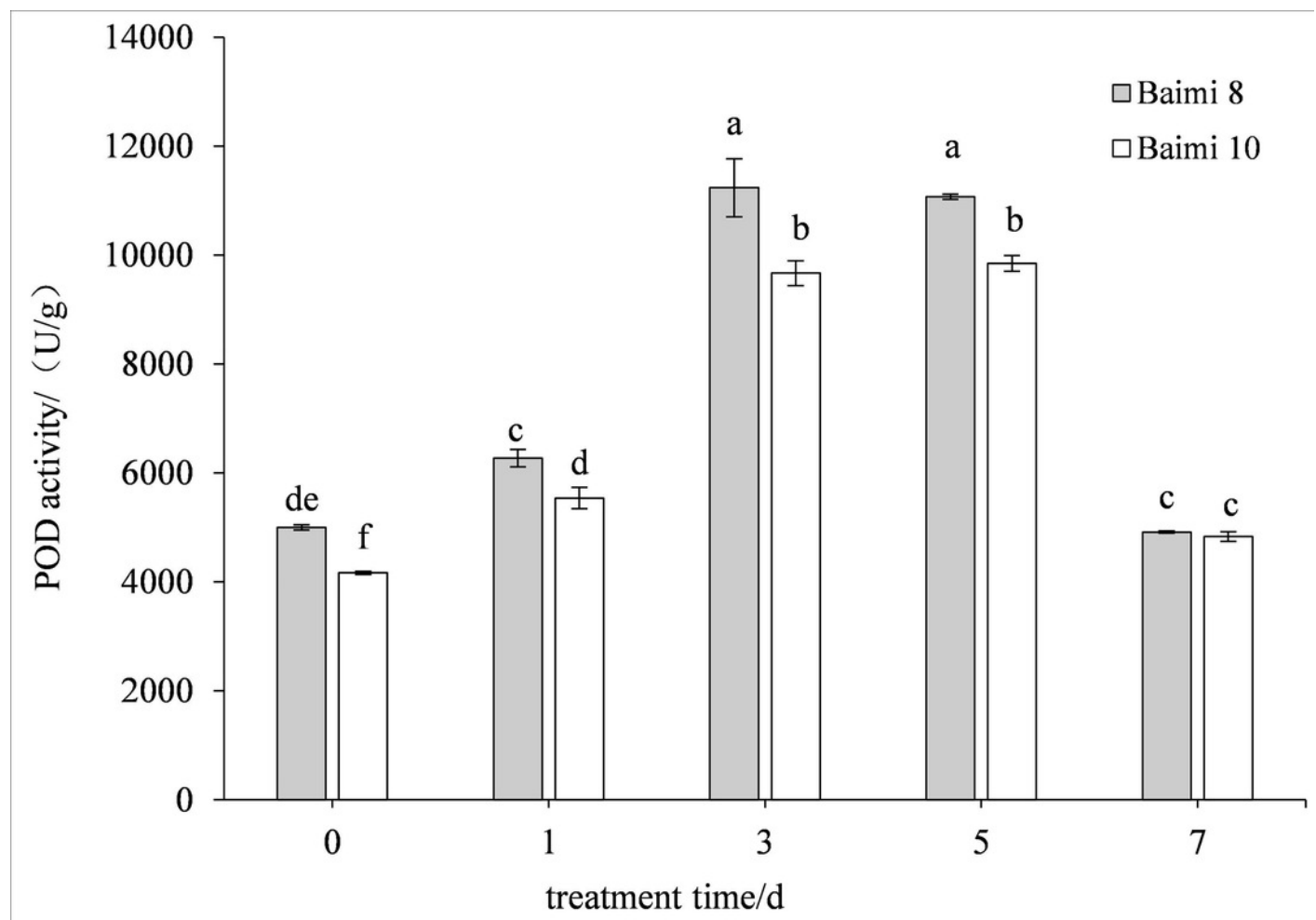
Figure 3

Response of catalase(CAT) to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of catalase activities significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
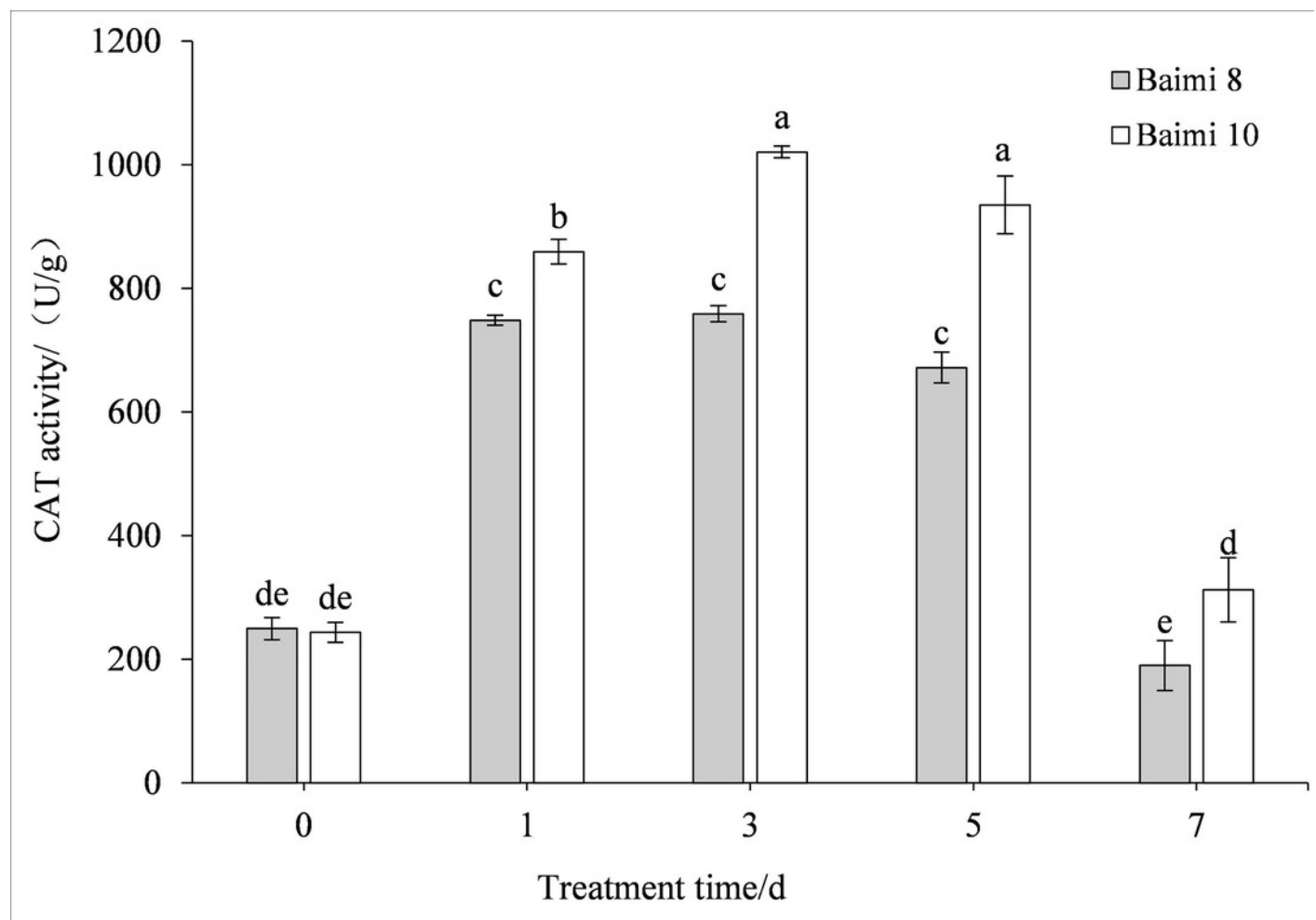
Figure 4

Response of ascorbic acid(APX) to waterlogging stress in pumpkin leaves

Different letters above columns indicate that the difference of ascorbic acid activities significant under the water logging (P < 0.05). Vertical bars = SD (n = 3)
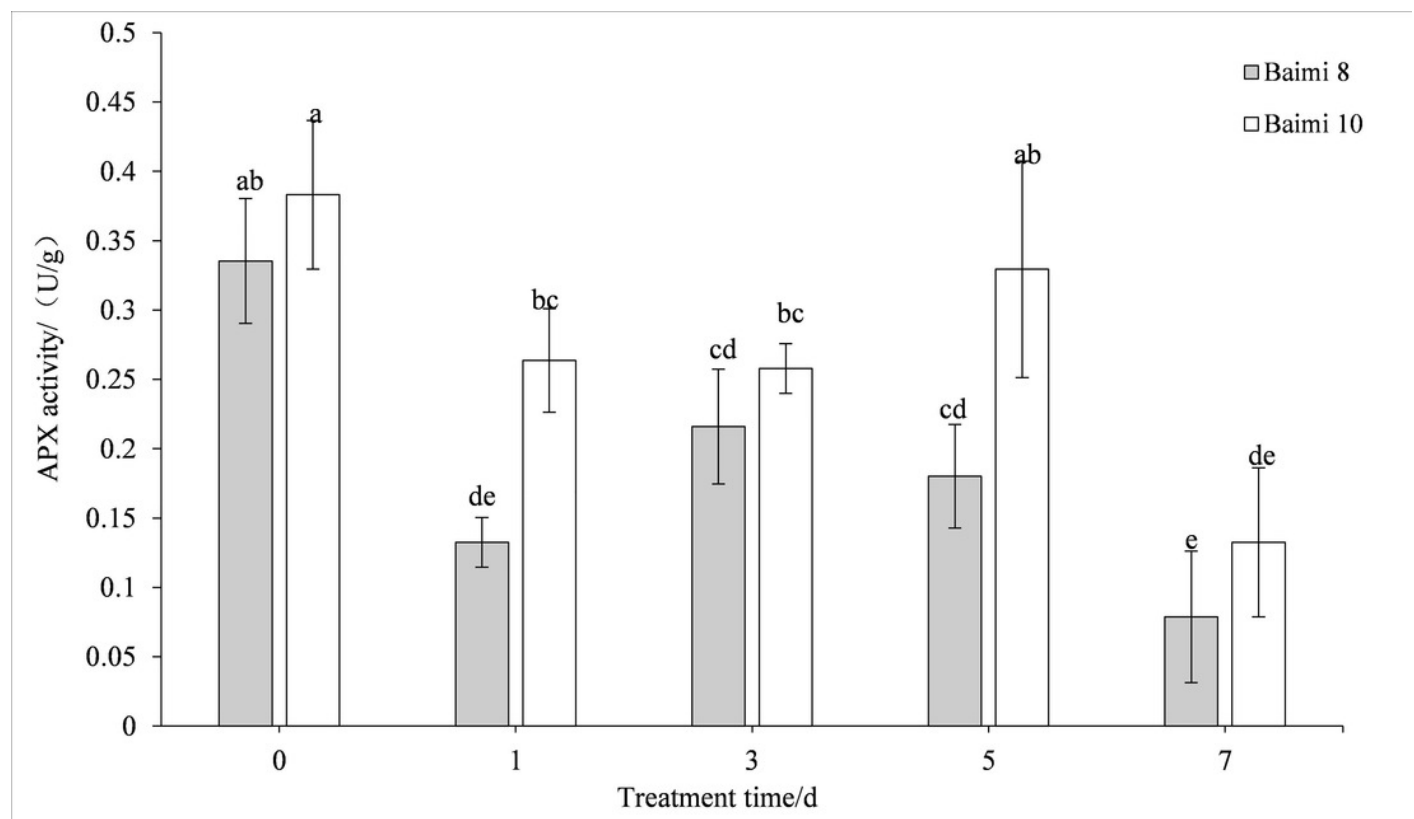
Figure 5

Response of malondialdehyde (MDA) to waterlogging stress in pumpkin leaves

Different letters above columns indicate that the difference of the content of malondialdehyde significant under the water logging (P < 0.05). Vertical bars = SD (n = 3)
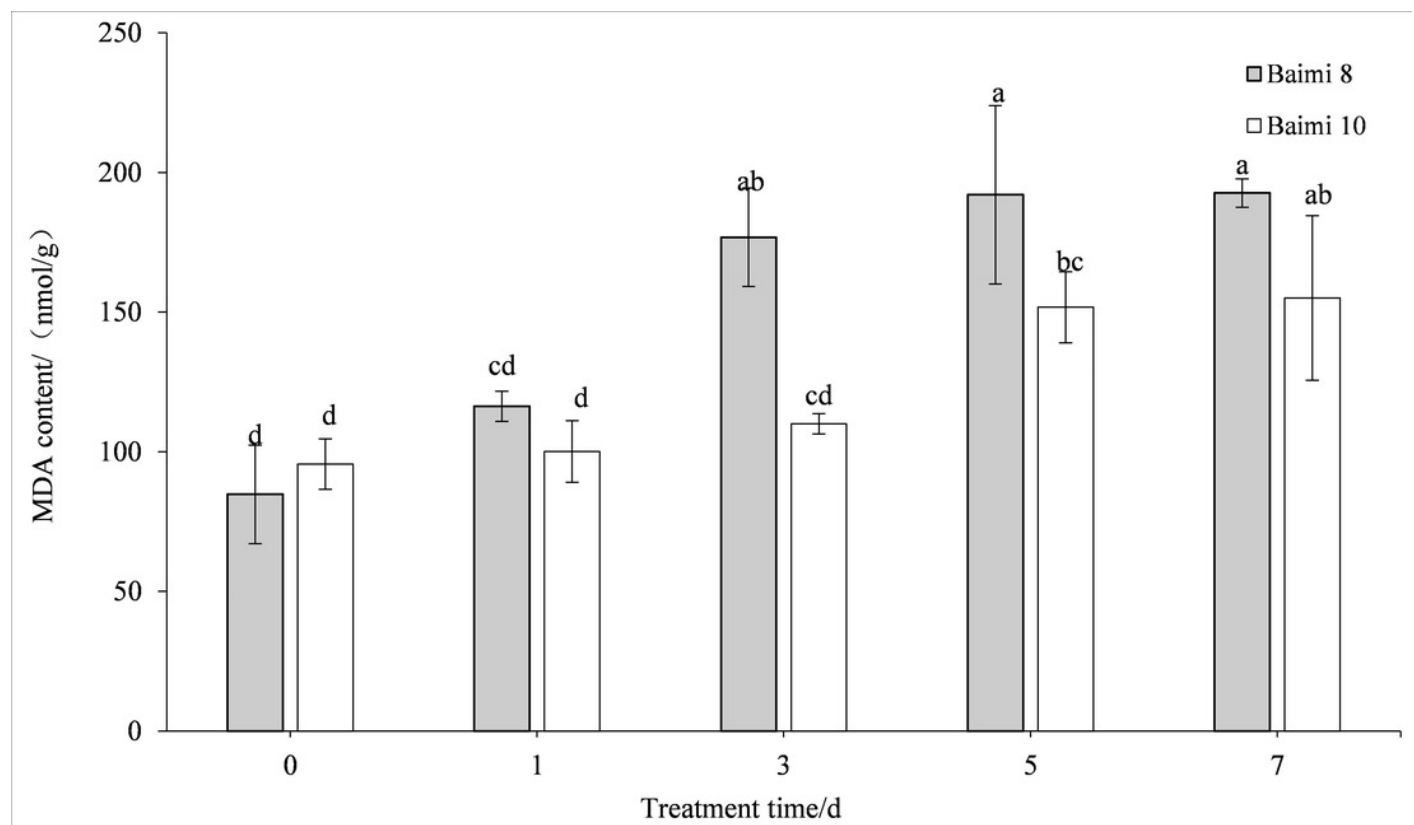
Figure 6

Response of proline(PRO)to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of the content of proline

significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
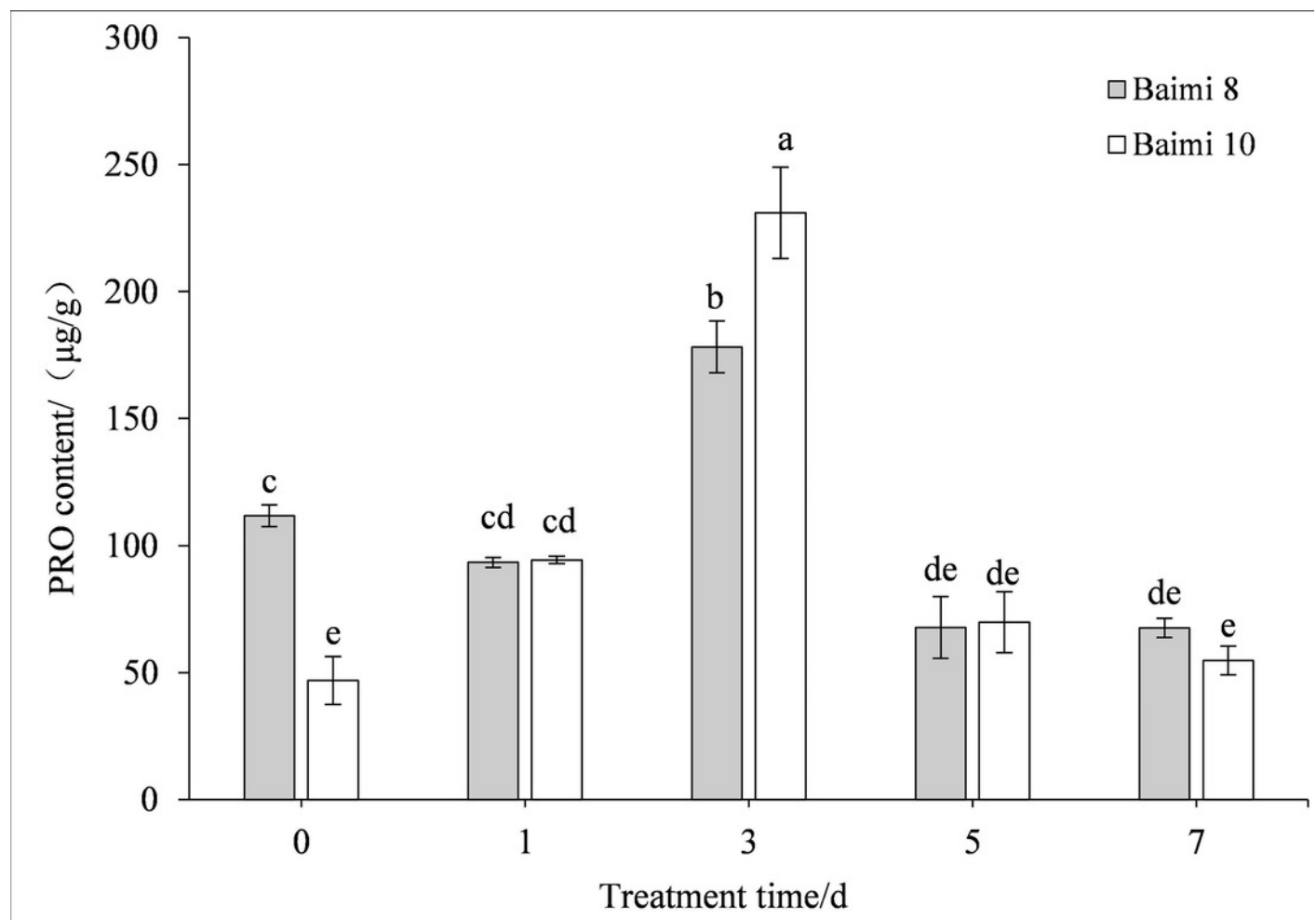
Figure 7

Response of alcohol dehydrogenase(ADH) to waterlogging stress in pumpkin roots.

Different letters above columns indicate that the difference of alcohol dehydrogenase(ADH) activity significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
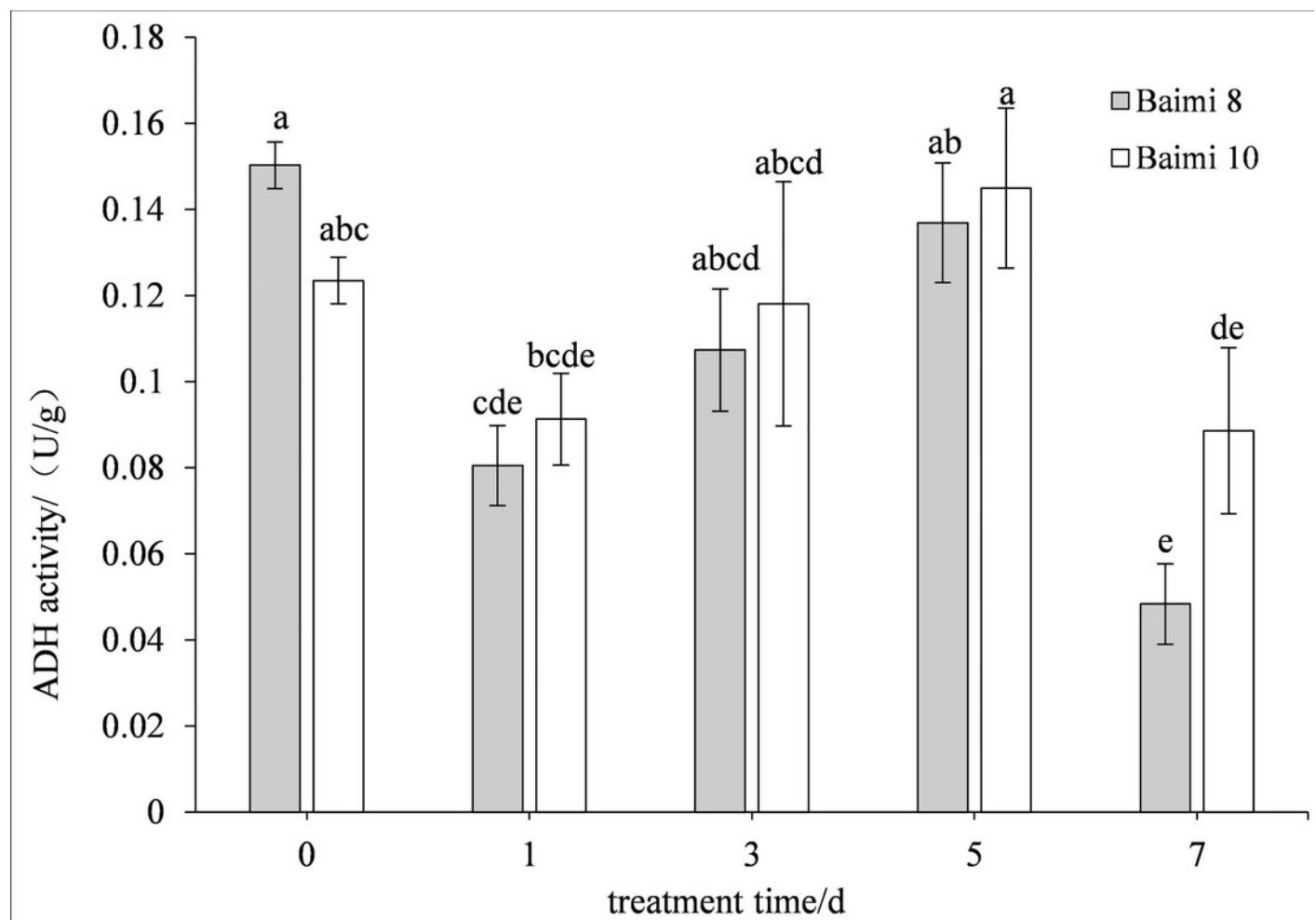
Figure 8

Response of pyruvate decarboxylase(PDC) to waterlogging stress in pumpkin roots.

Different letters above columns indicate that the difference of pyruvate decarboxylase activity significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
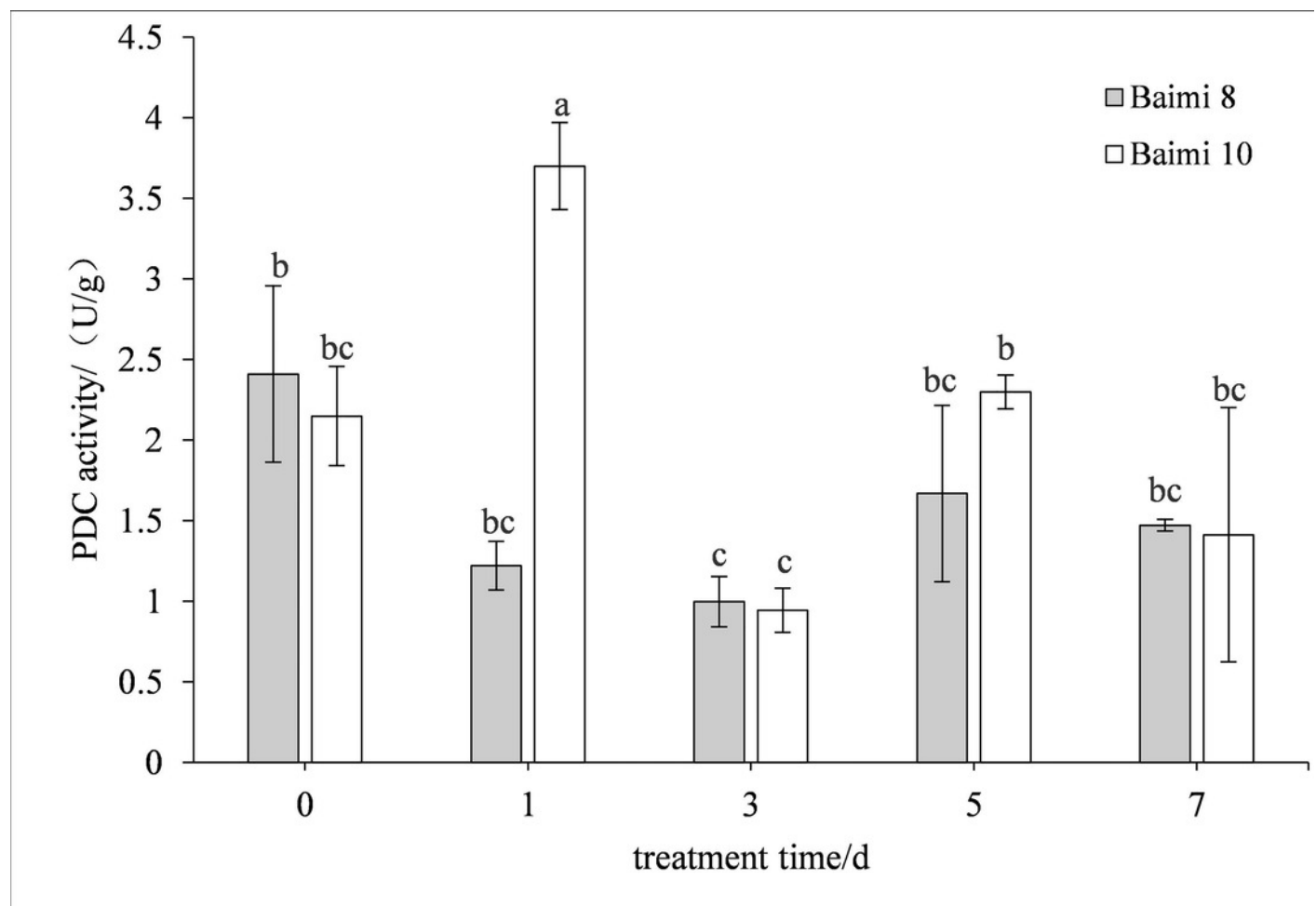
Figure 9

Response of gene expression of superoxide dismutase(SOD) to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of the gene expression of superoxide dismutase significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
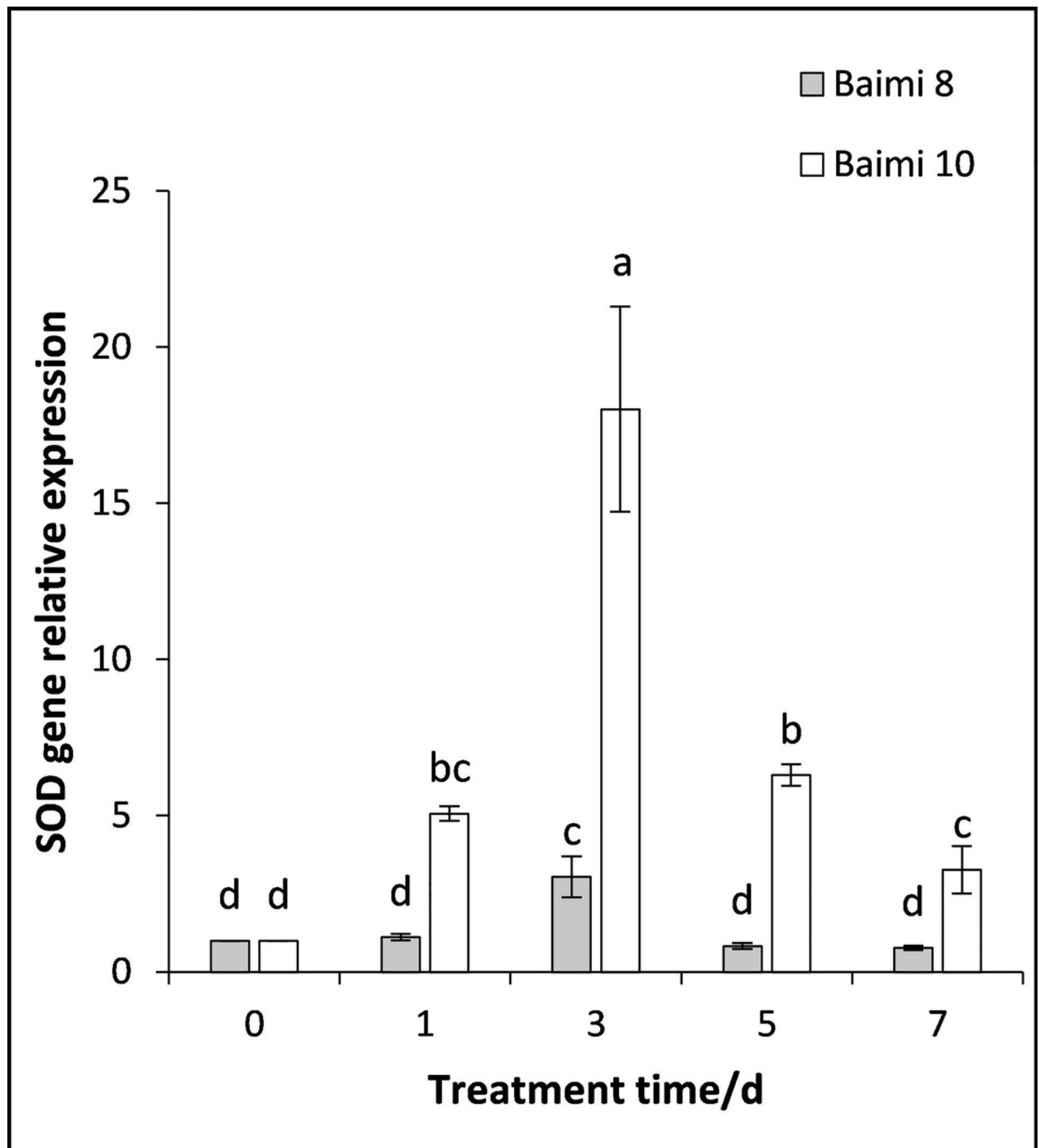
Figure 10

Response of gene expression of peroxidase(POD)to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of the gene expression of peroxidase significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
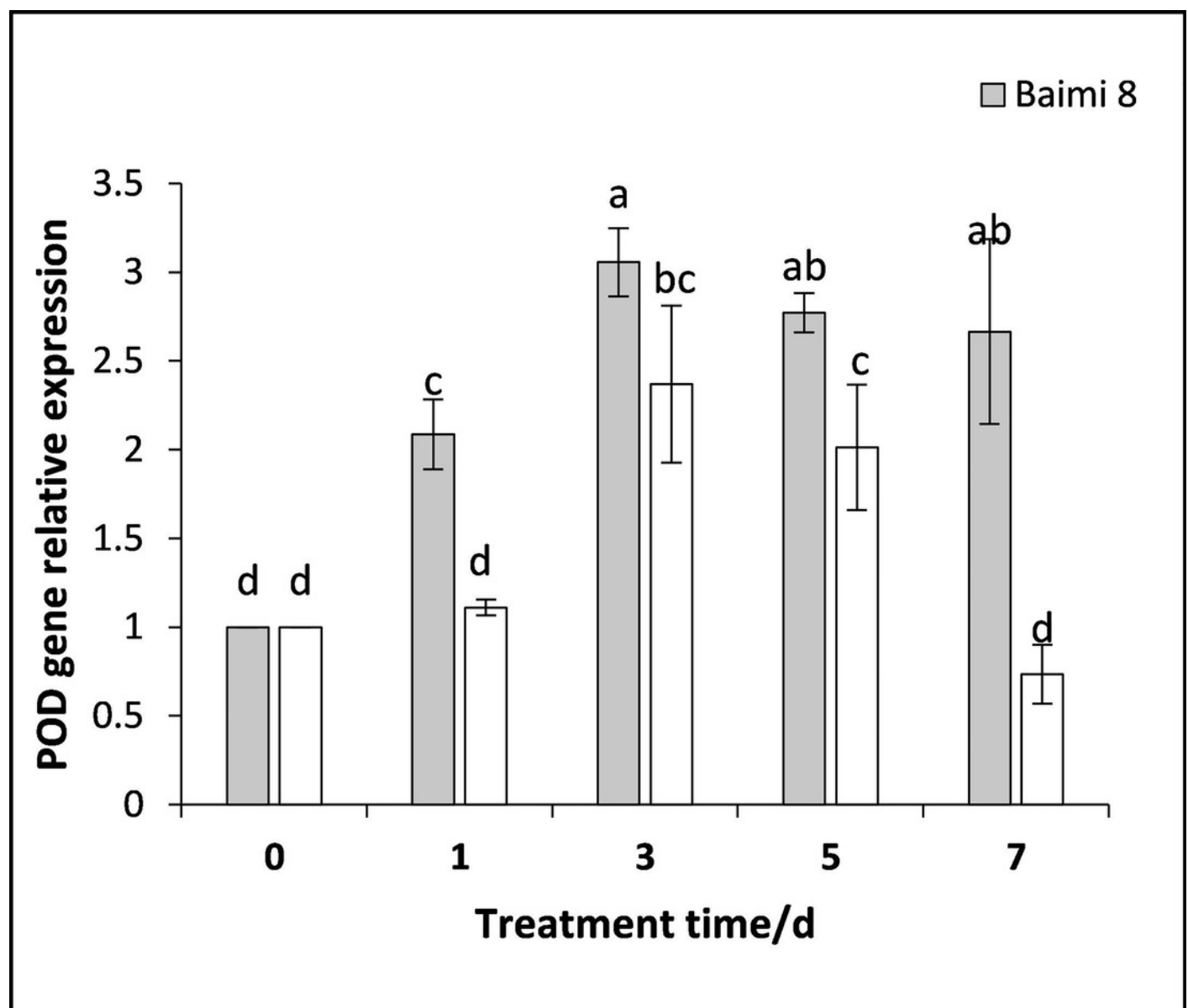
Figure 11

Response of gene expression of catalase(CAT) to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of the gene expression of catalase significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
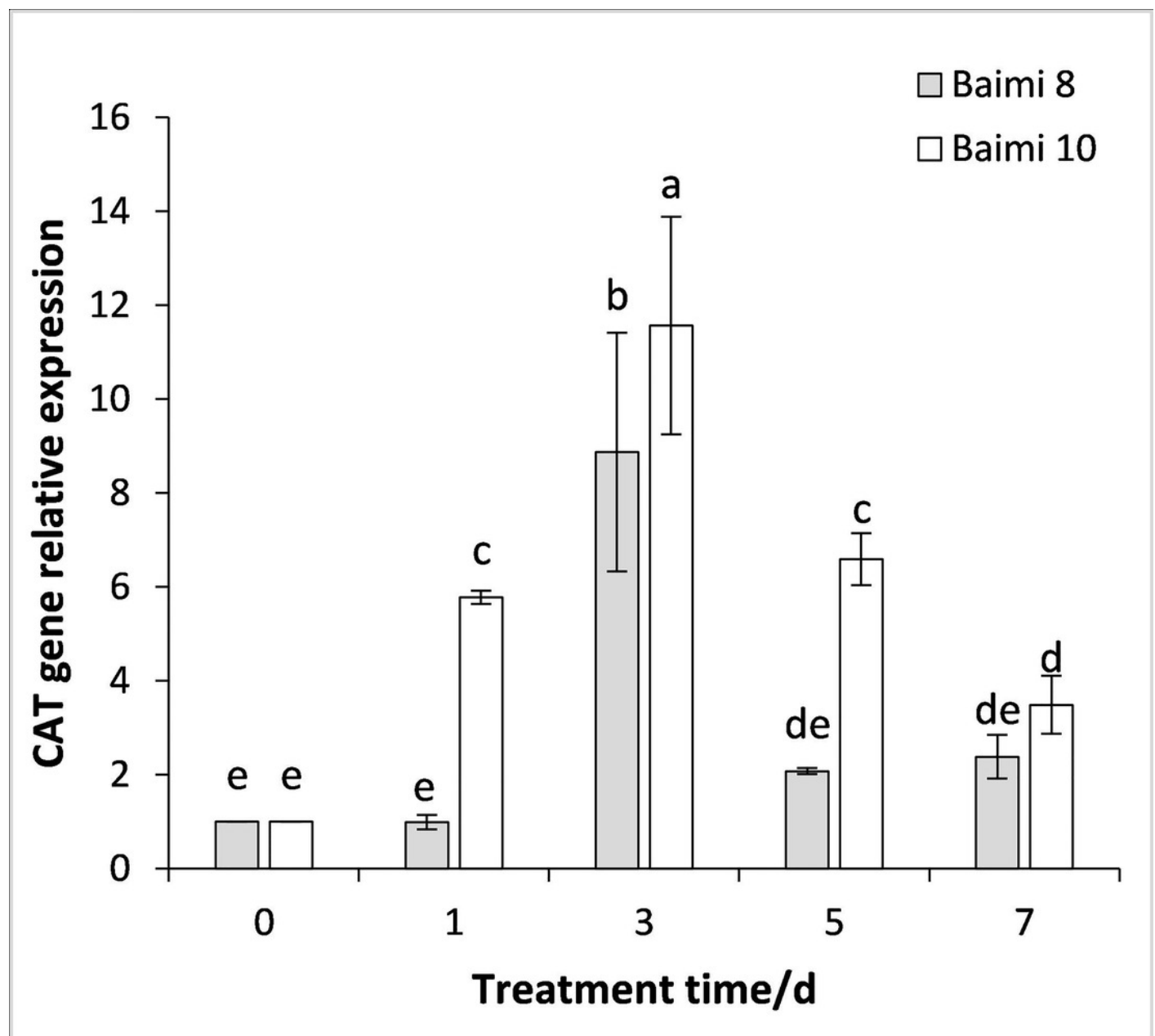
Figure 12

Response of gene expression of ascorbic acid(APX) to waterlogging stress in pumpkin leaves.

Different letters above columns indicate that the difference of the gene expression of ascorbic acid significant under the waterlogging (P < 0.05). Vertical bars = SD (n = 3)
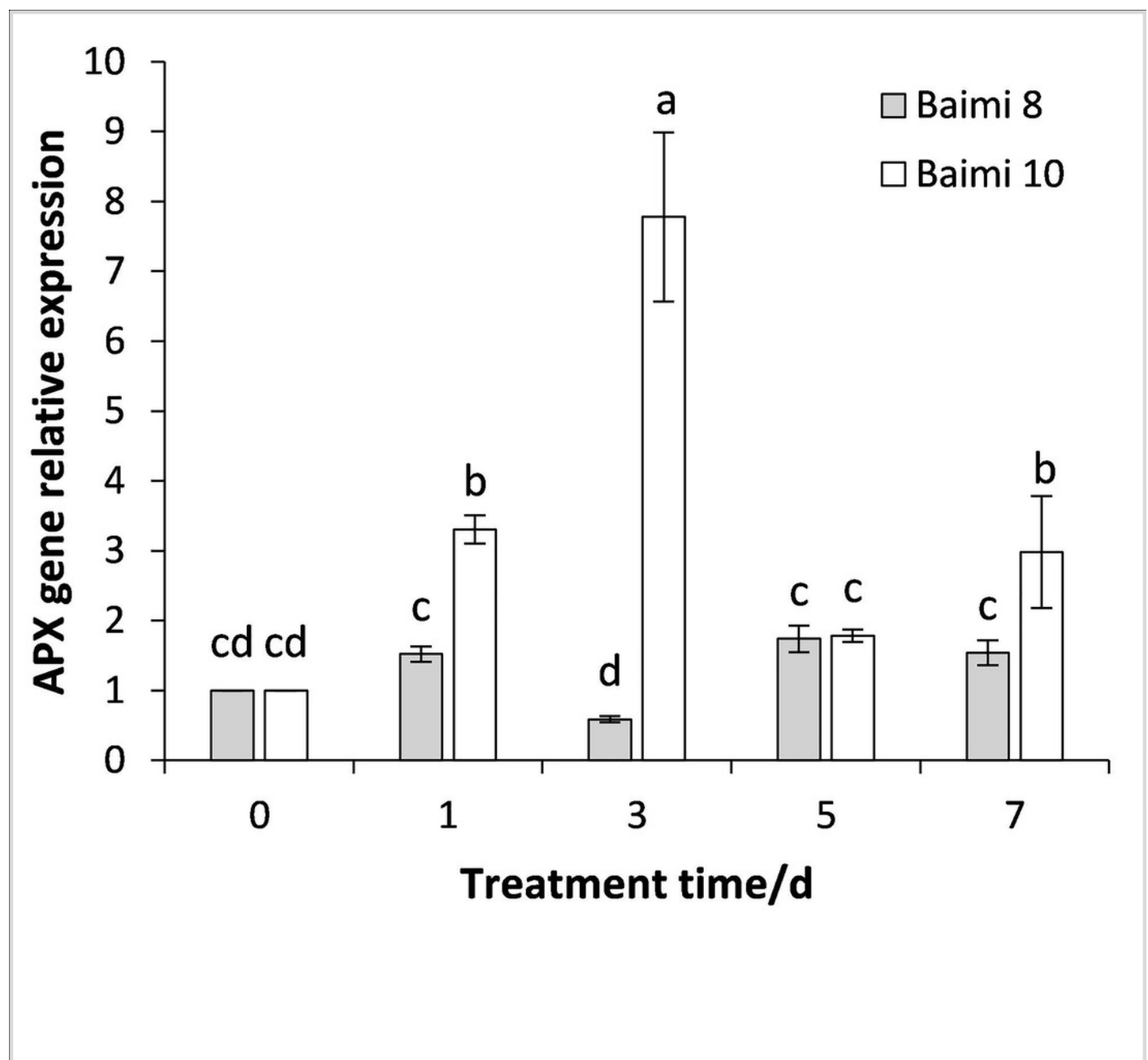
Table 1

Table 1 Retrotranscriptional response system

Reagent	System1	Reagent	System2
Template RNA∶Total RNA	2 μg	Template RNA Primer Mixture(come from system1)	10μl
Primer∶Oligo(dT) (50 uM)	1 μl	5×Reaction Buffer	4μl
dNTP Mix(10 mmol/L)	1 μl	RNase Inhibitor (40 U/μl)	0.5μl
		MMLV RT(200 U/μl)	1μl
		RNase free dH$_2$O	Up to 20μl

Table 1 Retrotranscriptional response system

Table 2

Primer sequences used for real-time fluorescence quantification

Table 2 Primer sequences used for real-time fluorescence quantification

The name of the gene	For/backward	Primer sequence (5' to 3')	The name of the gene	For/backward	Primer sequence (5' to 3')
β-action	F	TCTCTATGCCAGTGGTCGTA	CAT	F	CCGATGCCGCCTAATGTGTTGA
	R	CCTCAGGACAACGGAATC		R	CGAACCGCTCTTGCCTATCTGG
SOD	F	TCCTTGCCCGACCTCCCTTAT	APX	F	GGCGTTATCCGTCGTAGACACA
	R	GCCTCGTGAAGTTGCTCAAGAG		R	TGTGCCAGCGTCATGCCAAG
POD	F	TGCTGAACCCTGCCCATGTAGA			
	R	GGTGTACCACGGTCGTTCCTCA			

Table 3

qPCR response system

Table 3 qPCR response system

Reagent	System
2×SYBR real-time PCR premixture	10μl
PCR specific primer F of 10uM	0.4μl
PCR specific primer R of 10uM	0.4μl
cDNA	1μl
RNase free dH$_2$O	Up to 20μl

Table 4

Waterlogging resistance coefficient of pumpkin biomass and physiological indexes under the waterlogging

1   Table 4 Waterlogging resistance coefficient of pumpkin biomass and physiological indexes under the

2   waterlogging

Variety	Waterlogging resistance coefficient=Treatment measured value/contrast measured value								
	Above ground fresh mass	Underground fresh mass	Above ground dry mass	Underground dry mass	SOD	POD	CAT	MDA	SPAD
1	0.938±0.026ab	0.881±0.004b	0.902±0..086ab	0.853±0.028abc	0.173±0.023f	1.230±0.035cd	0.977±0.023cd	1.645±0.087a	0.933±0.002a
2	0.994±0.002a	0.858±0.02bc	0.856±0.060abc	0.792±0.029c	0.573±0.023e	1.313±0.038cd	1.397±0.017bc	1.159±0.024c	0.941±0.003a
3	0.965±0.012a	0.980±0.069a	0.909±0.011ab	0.943±0.07a	1.987±0.026b	1.633±0.058bc	0.593±0.020d	0.685±0.15e	0.805±0.010c
4	0.718±0.009f	0.784±0.01cd	0.742±0.027c	0.766±0.020c	0.233±0.07f	0.713±0.24ef	0.817±0.22cd	0.841±0.17d	0.714±0.004e
5	0.850±0.004cd	0.949±0.018ab	0.961±0.012a	0.917±0.019ab	1.130±0.023d	1.903±0.069b	1.040±0.46cd	1.485±0.54b	0.831±0.005bc
6	0.839±0.034d	0.781±0.040cd	0.749±0.022c	0.780±0.017c	0.660±0.17e	0.487±0.12f	0.663±0.44cd	1.547±0.20ab	0.954±0.006a
7	0.869±0.008cd	0.896±0.006ab	0.818±0.032bc	0.836±0.030bc	1.693±0.37c	0.810±0.66ef	1.893±0.347b	1.110±0.71c	0.852±0.022b
8	0.780±0.013e	0.736±0.16d	0.759±0.07c	0.772±0.016c	2.033±0.47b	0.937±0.07de	0.467±0.60d	0.523±0.33f	0.730±0.013de
9	0.943±0.026ab	0.859±0.27bc	0.809±0.45bc	0.853±0.022abc	1.727±0.38c	1.757±0.78b	0.387±0.30d	0.928±0.38d	0.845±0.004b
10	0.905±0.017bc	0.915±0.19ab	0.904±0.033ab	0.921±0.003ab	3.093±0.110a	5.443±0.395a	5.633±0.673a	1.130±0.18c	0.754±0.003d

3   Note:The data are mean ± SD (n=3).Different lowercase letter after the data in the same column indicate significant

4   differences among varieties(P<0.05).

Table 5

Membership function value and comprehensive evaluation value and ranking of pumpkin varieties

1   Table 5 Membership function value and comprehensive evaluation value and ranking of pumpkin varieties

Variety	PC_1	PC_2	PC_3	MF(X_1)	MF(X_2)	MF(X_3)	CEV	Rank
1	-0.016	2.239	0.493	0.424	1.000	0.685	0.649	3
2	0.015	1.700	0.064	0.429	0.883	0.573	0.598	4
3	1.938	-0.124	-2.126	0.721	0.489	0.000	0.546	5
4	-2.802	-1.534	0.160	0.000	0.185	0.598	0.143	9
5	1.502	0.946	-0.262	0.655	0.721	0.487	0.653	2
6	-2.146	1.300	1.146	0.010	0.797	0.855	0.433	8
7	-0.046	-0.014	-0.023	0.419	0.513	0.550	0.468	6
8	-2.213	-2.388	-0.464	0.090	0.000	0.435	0.108	10
9	0.001	-0.013	-0.686	0.427	0.513	0.377	0.448	7
10	3.768	-2.113	1.699	1.000	0.059	1.000	0.692	1

2   The eigenvector expression is:

3   PC1=0.303X1＋0.420X2＋0.416X3＋0.444X4＋0.300X5＋0.400X6＋0.326X7＋0.066X8＋0.019X9;

4   PC2=0.333X1＋0.172X2＋0.191X3＋0.031X4－0.405X5－0.234X6－0.222X7＋0.577X8＋0.470X9;

5   PC3=－0.195X1－0.290X2－0.158X3－0.236X4－0.057X5＋0.359X6＋0.590X7＋0.540X8＋0.159X9;

6   PC1:the first principal component;PC2:the second principal component;PC3:the third principal component.

7   X1:Above   ground   fresh   mass;X2:Underground   fresh   mass;X3:Above   ground   dry   mass;X4:Underground   dry

8   mass;X5:SOD;X6:POD;X7:CAT;X8:MDA;X9:Chlorophyll SPAD content.