

1 Effects of melatonin on germination and amino acid
2 content in different wheat varieties seeds under
3 polyethylene glycol stress

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11 **Abstract**

12 In this research, the effects of melatonin on germination and amino acid content in
13 wheat (*Triticum aestivum* L.) seeds under polyethylene glycol (PEG-6000) stress were
14 investigated. The results revealed that 10 μ M melatonin could aggravate the adverse
15 effects of drought stress on germination percentage, germination index, and
16 germination potential of two wheat varieties (JM22 and HG35). However, 300 μ M
17 melatonin could obviously alleviate the negative effect of water stress on germination
18 and increase radicle length, radicle number and plumule length of germinated seeds.
19 Principal component analysis showed that amino acid content apparently changed in
20 germination and the variation amplitude was different due to melatonin concentrations
21 and wheat varieties. Lys content in wheat seed under 300 μ M melatonin plus PEG
22 treatment increased compared with that under PEG. Meanwhile, Lys content was
23 significantly and positively correlated with germination percentage, germination
24 index, germination potential, radicle length, and plumule length, respectively. Our
25 results suggested Lys play an important role in the regulation of melatonin on drought

26 resistance of wheat seed during germination. This may be of value for saving water
27 resources in North China Plain.

28 **Introduction**

29 Drought is a major abiotic stress that lead to great loss in agriculture worldwide.
30 In North China Plain, wheat production has been enduring severely constraint such as
31 yield increasing, quality improving, and groundwater exploitation decreasing.
32 Meanwhile, effective rainfall is scarce for meeting needs of plants during wheat
33 growth stage [1]. As food crop, it is urgent to ensure wheat supply safety for the
34 rapidly increasing world population. Melatonin, a derivative of the essential amino
35 acid tryptophan, can trigger the plant defense responses against adverse environment
36 stress [2-4]. The possible protective roles of melatonin, such as abiotic anti-stressor,
37 biotic anti-stressor, biological rhythm regulator, plant (hormone) regulator and so on,
38 are increasingly curious and researched [5-8].

39 Previous studies had pointed out that melatonin applied to cucumber seeds could
40 improve their germination rate during chilling stress and water-stress with respect to
41 untreated seeds [4,9]. The pretreatment of seeds with melatonin reduced copper
42 toxicity in red cabbage seedlings (*Brassica oleracea rubrum*) [10]. As is well-known,
43 amino acid metabolism was related and provided nutrient during seed germination
44 [11]. Melatonin share common precursors in the biosynthetic route of tryptophan and
45 tryptamine [12]. Melatonin pretreatment resulted in the osmoprotection through the
46 regulation of proline homeostasis and the enhancement of plant tolerance to drought
47 conditions [13]. A new research reported that melatonin alleviated the inhibitory

48 effects on storage protein degradation of cucumber seed under salt stress, which
49 possible related to amino acid content changing [14].

50 Additionally, different concentrations melatonin had taken different effects on
51 seeds germination and seedling growth [15]. 1 or 10 μ M melatonin could eliminate
52 the inhibitory effect of copper on the fresh weight of seedlings. But 100 μ M
53 melatonin had a negative effect on seed germination, seedling grown, and even
54 enhanced the toxic effect of copper [10]. Maize seed priming with 0.8 mM melatonin
55 significantly improved germination energy, germination percentage, proline and total
56 phenolic contents [16]. So far, there are insufficient data on how different
57 concentrations of melatonin affect wheat seed germination by amino acid changing
58 for different wheat varieties.

59 The main aims of this article were: (1) to investigate the germination
60 characteristics of two wheat varieties at different levels of melatonin over drought
61 conditions; (2) and to evaluate changes in amino acid content during germination of
62 wheat seed. This would help to obtain exact data for further use in wheat yield
63 protection during drought resistance.

64 **Material and methods**

65 **Tested materials and reagents**

66 The experiment was carried out in a key laboratory of crop growth regulation,
67 Agricultural University of Hebei, Baoding city, China in 2017. Two wheat (*Triticum*
68 *aestivum* L.) cultivars including the drought-tolerant cultivar ‘Hengguan35’ (HG35)
69 and the irrigated cultivar ‘Jimai22’ (JM22) were used in this study. The HG35 seeds

70 were provided by Dry Land Farming Research Institute of Hebei Academy of
71 Agricultural and Forestry Sciences, whereas the cultivar JM22 seeds were donated by
72 Crop Research Institute, Shandong Academy of Agricultural Sciences. The melatonin
73 and polyethylene glycol 6000 (PEG6000) used in the study were purchased from
74 Beijing Sinopharm Chemical Reagent Co., Ltd.

75 **Experimental methods**

76 **Preparation of melatonin solution**

77 Firstly, distilled water was used as the basic medium to prepare a 20% PEG solution.
78 Secondly, melatonin was firstly diluted by 1mL 95% ethyl alcohol and then was
79 added to 20% PEG solution to prepare 0, 1, 10, 100 and 300 $\mu\text{mol/L}$ melatonin
80 solutions.

81 **Experimental treatment**

82 Big and full wheat seeds were selected and sterilized in surface with 70 percent
83 ethanol for two minutes. Then seeds were washed several times with distilled water
84 and were put into the same germination box, in which two-layer filter paper were
85 fully saturated by distilled water (control, CK), polyethylene-glycol solution (20%
86 PEG6000), 20% PEG plus 1 $\mu\text{mol}\cdot\text{L}^{-1}$ melatonin solution (1 μM +20% PEG), 20%
87 PEG plus 10 $\mu\text{mol}\cdot\text{L}^{-1}$ melatonin solution (10 μM +20% PEG), 20% PEG plus 100
88 $\mu\text{mol}\cdot\text{L}^{-1}$ melatonin solution (100 μM +20% PEG), and 20% PEG plus 300 $\mu\text{mol}\cdot\text{L}^{-1}$
89 melatonin solution (300 μM +20% PEG), respectively. There were six treatments with
90 three repeats. Each germination box was posed 50 wheat seeds and placed in an
91 incubator at fluctuating day/night temperatures of 20°C/15°C in a light/dark regime.

92 Seed germination was counted daily up to 7 days after placement in the incubator.

93 Water and each concentration solution were complemented into these boxes in time.

94 **Measurements**

95 The germination percentage was a proportion of emerged- germinating seed in total

96 cultivated seed on 7 days after placement in the incubator.

97 The germination potential was the germination percentage on 3 days after
98 placement in the incubator.

99 Root length, sprout length, and radical length of germinated seed were measured
100 5 repeats with ruler began from 72 hours after placement in the incubator.

101 Vigor index was measured according to Lu et al. [17].

102 Vigor index= The germination percentage \times radical length

103 Measurement of amino acid content: Wheat seeds were milled to a fine powder
104 and sifted through 100 mesh. Flour samples (1.00 g) were hydrolyzed for 14 hr at
105 110°C in the presence of 10 mL of 6 mol·L⁻¹ hydrochloric acid. Then it was diluted
106 with water to 10 mL at room temperature. About 1 mL extractions were transferred
107 into centrifuge tube to be concentrated and dried with vacuum chamber. Then the
108 concentrated sample were dissolved with 2 mL hydrochloric acid (0.1 mol·L⁻¹). And
109 the dilute sample solution was determinate by reversed-phase high performance liquid
110 chromatograph (HPLC). The HPLC system (Agilent 1200, USA) consisted of a C18
111 column with 5 μ m particle size (4.6 mm inner diameter, 250 mm length, Komati
112 Universal) and a diode array detector. The mobile phase A was prepared by adding
113 acetonitrile. The mobile phase B was prepared by adding acetic acid-sodium acetate

114 buffer (PH 5.25±0.05, glacial acetic acid adjusting) containing 0.03 mol·L⁻¹ sodium
115 acetate solution and 0.15% triethylamine. The column temperature was 40°C. The
116 determine wavelength was 360 nm. The flow rate was 1 mL·min⁻¹ at ambient
117 temperature. Automatic injection of as many as 42 samples was realized by a G1329A
118 injector with a 10-μL sample loop. The gradient elution procedures were showed at
119 Table 1.

120 **Table 1. Gradient elution program**

Time/min		0	10	12	25	30	37	39	45
Mobile phase	A	18	18	29	34	55	60	18	18
	B	82	82	71	66	45	40	82	82

121 Aminoacyl derivatization: 100 μL preparing standard solutions or sample
122 solutions were transferred into 1.5 ml centrifuge tube by adding 200 μL buffered
123 solution (pH 9.0) and 100 μL 2,4-dinitrochlorobenzene (300 mg·mL⁻¹, acetonitrile as
124 solvent). The mixture was vortex-mixed for 1 min and extracted in a 90°C thermostatic
125 water bath for 90 min in dark. After the reaction had finished, the resulting solution
126 was added by 50 μL 10%(v/v) acetic acid, adjusted neutral pH value, and diluted the
127 volume to 1mL with distilled water. The solutions were filtered through the organic
128 membrane.

129 Standard Curve Development: Standard solutions (500 mg·L⁻¹) of 17 amino acid
130 including aspartic acid (Asp), glutamic (Glu), histidine (His), serine (Ser), arginine
131 (Arg), glycine (Gly), threonine (Thr), proline (Pro), alanine (Ala), valine (Val),
132 methionine (Met), cysteine (Cys), isoleucine (Ile), leucine (Ile), phenylalanine (Phe),
133 lysine (Lys), and tyrosine (Tyr) were prepared by dilution of a stock solution (amino

134 acid standard mixture, 10 ml, 0.1 mol·L⁻¹ HCl). Those were diluted to 5、 50、 125、
135 100、 150、 200、 300、 400、 500 mg·L⁻¹ with 0.1 mol·L⁻¹ HCl. At last, 100 μL preparing
136 standard solutions were measured after aminoacyl derivatization performed as above.

137 **Data processing**

138 All data were run using analysis of variance (ANOVA) with three replicates
139 according to Excel 2010 and SPSS 19.0 (SPSS Inc., Chicago, USA). The Duncan's
140 new multiple range (DMR) test at 5% probability level was used to test the
141 differences among the mean values.

142 **Results**

143 **Effects of different concentrations of melatonin on statistical** 144 **germination**

145 For JM22, germination percentage and germination index decreased significantly by
146 14.29% and 93.27% under PEG treatments compared with that under control,
147 respectively (Table 2). And 1, 10, 100 μM melatonin plus PEG solution all did not
148 improve germination percentage and germination index. Even 10 μM+PEG treatment
149 reduced more on the two values relative to that of PEG. However, germination
150 percentage could be improved significantly under 300 μM+PEG treatment comparing
151 with that under other treatments. There was no significant difference on germination
152 percentage between control and 300 μM+PEG treatment. But germination index of
153 seed under 300 μM+PEG treatment decreased significantly comparing with the
154 control. Additionally, 1 and 10 μL melatonin plus PEG treatments significantly

155 decreased germination potential; whereas it was not changed obviously among other
156 treatments.

157 For HG35, compared with the control, germination percentage and germination
158 potential under PEG treatment were both not significantly decreased; but germination
159 index decreased by 50.48% ($P < 0.05$). $10\mu\text{M} + \text{PEG}$ treatment further decreased
160 germination percentage significantly. Compared with PEG, germination index under
161 $100\mu\text{M} + \text{PEG}$ and $300\mu\text{M} + \text{PEG}$ treatments increased by 29.81% and 34.50%,
162 respectively ($P < 0.05$).

163 **Table 2. The effect of different treatments on the germination of wheat seeds**

Treatments	JM 22			HG35		
	Germinatio n percentage	germinatio n index	germinatio n potential	Germinatio n percentage	germinatio n index	germinatio n potential
Control	96.0a	120.6a	96.0a	98.7a	125.2a	98.7a
PEG	84.0b	62.4c	80.0ab	95.3abc	83.2d	94.7ab
$1\mu\text{M} + \text{PEG}$	78.0b	72.3c	76.7b	88.7cd	97.1c	88.7bc
$10\mu\text{M} + \text{PEG}$	66.7c	37.7d	43.3c	82.7d	81.3d	80.7bc
$100\mu\text{M} + \text{PE}$						
G	77.3b	76.4c	77.3ab	90.0bcd	108.0bc	89.3b
$300\mu\text{M} + \text{PE}$						
G	99.3a	100.1b	94.0ab	96.7ab	111.9b	88.7bc

164 The same lower case letters indicate no significant difference ($P < 0.05$)

165 **Effects of different concentrations of melatonin on morphological**
166 **characters of germination**

167 In this study, radicle length and radicle number of two wheat cultivars were both
168 increased gradually from 72 hours to 144 hours after germination (Fig 1). For JM22,
169 radicle length under PEG and $10\mu\text{M} + \text{PEG}$ treatments decreased by 131.00% and
170 189.00% on average compared with the control. No obvious change had been found
171 on that under $1\mu\text{M} + \text{PEG}$ and $100\mu\text{M} + \text{PEG}$ treatments. However, $300\mu\text{M} + \text{PEG}$

172 treatment resulted in an increase of 79.01% on radicle length compared with that
173 under PEG treatment. Compared with the control, radicle number under other
174 treatments were decreased significantly; and the least value under 10 μ M+PEG
175 treatments decreased by 79.6% ($P<0.05$). Under 300 μ M+PEG treatment, radicle
176 number increased by 24.41% compared with that under PEG treatment. And the
177 plumule length was decreased significantly under all other treatments compared with
178 the control. There was hardly obvious improving effect of melatonin on plumule
179 length under PEG treatment.

180 For HG35, compared with the control, radicle length and plumule length were
181 both decreased significantly under all other treatments. Radicle length of wheat seed
182 under 300 μ M melatonin plus PEG treatment decreased by 85.72% compared with the
183 control; but it increased by 20.65% compared with PEG treatment. There was no
184 significant difference on radicle number between control and 300 μ M+PEG treatment.
185 The changing trend of plumule length was similar to that of JM22. Additionally, as a
186 drought-resistant variety, radicle length and radicle number of HG35 were both higher
187 than that of JM22 under PEG treatment.

188 **Fig 1. Effects of different concentrations of melatonin on radicle length, radicle**
189 **number, and plumule length of JM22 and HG35.** Values are means of three
190 biological replications.

191 **Effects of different concentrations of melatonin on amino acid** 192 **content**

193 The results showed the variations among all different treatments (Fig 2). Principal
194 component analysis extracted two major components that accounted cooperatively for

195 74.8% of the variance in the data set. Principal component 1 (PC1, X-axis) explained
196 43.8% of the variation among the individual samples, principal component 2 (PC2,
197 Y-axis) explained 31.0% of the variation. When seed germinated, the amino acid
198 content of both cultivars shift greatly along PC2 axis. This meaning that amino acid
199 content apparently changed in germination. When seed germinated with PEG-6000,
200 the amino acid content of both cultivars goes down along PC2 axis; moreover, HG35
201 and JM22 were separated with each other. This indicated a genotypic variation of
202 amino acid content in seed germination with PEG-6000. This may be the reason why
203 seed germination of HG35 was better than that of JM22 under water stress condition.

204 **Fig 2. Principal component analysis of amino acid for different wheat cultivars.**
205 Germination represent dry seeds germinated by distilled water; PEG represent dry seeds
206 germinated by 20% PEG; Seed represent dry seeds.

207 Glu content was the highest in wheat seed of both cultivars, followed by Pro
208 content. After seed germinated, Glu and Pro content reduced by 17.16 mg kg⁻¹ and
209 4.41 mg kg⁻¹ for JM22; and those reduced by 12.96 mg kg⁻¹ and 3.56 mg kg⁻¹ for
210 HG35, respectively (Table S1). This indicated that Glu and Pro could be the source of
211 nitrogen during quiescent dry seed resuming metabolic activity. After germinated, Lys
212 and Tyr contents in CK seeds both increased by 129.22% and 130.77% for JM22; and
213 those increased by 71.85% and 98.59% for HG35, respectively. But comparing with
214 CK, Lys and Tyr contents in seeds under other treatments were all decreased at
215 different levels; Glu and Pro contents were reversely increased (Fig 3). The variation
216 amplitude of amino acid content was different due to melatonin concentrations and
217 wheat varieties. For JM22, comparing with CK, Lys and Tyr content reduced by

218 48.44% and 36.67% in seed under PEG; melatonin plus PEG treatments raised the
219 reduction except 300 μ M melatonin reducing Lys content by 37.96%. Compared with
220 CK, Glu and Pro content increased by 44.41% and 29.52% in seed under PEG; and
221 the two indexes increased by 74.40% and 48.19% on average under PEG plus low
222 concentration melatonin (1 and 10 μ M). For HG35, comparing with CK, Lys and Tyr
223 content reduced by 62.72% and 53.19% under PEG; Lys content reduced by 57.97%
224 and 39.87% under melatonin plus 100 μ M and 300 μ M treatments, respectively. On
225 the same situation, Glu and Pro content increased by 51.97% and 55.27% in seed
226 under PEG; the increasing amplitude of the two indexes decreased under PEG plus
227 different concentrations melatonin treatments.

228 Compared with PEG treatment, the contents of Glu, Met, Cys, and Tyr in JM22
229 seed decreased under 300 μ M melatonin plus PEG treatment, but other amino acid
230 contents increased (Fig 3 and Table 1S). Cys and Lys content in HG35 seed under
231 300 μ M melatonin plus PEG treatment was higher than that of PEG treatment,
232 whereas other components was lower.

233 **Fig 3. The cluster heatmap of amino acid content differences of two wheat**
234 **varieties during germination under different treatments.** The graph's horizontal axis
235 shows different treatments (seed represent no-germinated seeds; CK represent seeds
236 germinated by distilled water; PEG represent seeds germinated by 20%PEG; 1 μ M, 10 μ M,
237 100 μ M, and 300 μ M, represent seeds germinated by 1 μ M, 10 μ M, 100 μ M, and 300 μ M
238 melatonin plus 20%PEG, respectively), and the vertical axis shows different amino acid.
239 Color gradients represent the differences value of amino acid contents under other treatments
240 compared with that of CK.

241 **Table S1 Effect of different concentrations of melatonin on amino acid content**
242 **in winter wheat under PEG treatments**

243

244 **Correlation between amino acid content and morphological indexes**

245 Correlation coefficients revealed that Asp content was significantly and positively
 246 correlated with germination index, radical length, and radical number (Table 3). Glu
 247 content was negatively correlated with all morphological indexes of germination,
 248 especially plumule length. After seed germinated, Glu and Pro content reduced
 249 significantly (Table S1 and Fig 3). This indicated that Glu and Pro possibly provided
 250 amino (NH_4^+) to other amino acid by aminotransferase when seed germinated. Phe
 251 content was significantly and positively correlated with radical number. And Lys
 252 content was significantly and positively correlated with germination percentage,
 253 germination index, germination potential, radicle length, and plumule length,
 254 respectively. This suggested that Lys played an important role in wheat germination
 255 and had an obvious interaction with melatonin.

256 **Table 3. The correlation analysis of variance between morphological index of**
 257 **germination and 17 amino acids**

Amino acid	Germination percentage	Germination index	Germination potential	Radicle length	Plumule length	Radicle number
Asp	0.333	0.602*	0.418	0.588*	0.501	0.732**
Glu	-0.364	-0.434	-0.357	-0.497	-0.643*	-0.224
His	-0.175	0.028	-0.14	-0.021	-0.105	0.259
Ser	-0.259	-0.231	-0.263	-0.294	-0.427	-0.042
Arg	-0.028	0.294	0.172	0.28	0.168	0.51
Gly	0.011	0.172	0.067	0.102	-0.042	0.406
Thr	-0.287	-0.196	-0.368	-0.21	-0.252	-0.035
Pro	-0.427	-0.538	-0.525	-0.531	-0.531	-0.371
Ala	0.049	0.161	0.077	0.182	0.105	0.35
Val	0.126	0.217	0.147	0.203	0.098	0.434
Met	-0.459	-0.305	-0.267	-0.287	-0.371	-0.091
Cys	0.084	0.231	0.252	0.091	-0.189	0.308
Ile	0.042	0.161	0.063	0.126	0.049	0.42
Leu	-0.042	0.098	-0.004	0.077	-0.042	0.336
Phe	0.280	0.413	0.414	0.410	0.305	0.585*
Lys	0.599*	0.609*	0.584*	0.673*	0.680*	0.522
Tyr	0.278	0.176	0.340	0.162	0.246	0.165

258 * indicate significant correlation ($P > 0.05$)

259 **Discussion**

260 Different concentrations of melatonin have taken different effects on crop growth
261 [18-19]. Melatonin (0-1 μM) promoted the root growth; but the higher melatonin
262 concentrations (5-10 mM) inhibited root growth and chlorophyll concentration of
263 cherry rootstock PHL-C [20]. In the present study, 10 μM melatonin could aggravate
264 drought stress effect on wheat seed germination under PEG condition. It has been
265 reported that exogenous melatonin with low concentration (1 μM and 10 μM)
266 inhibited leaf physiology by communication of abscisic acid and hydrogen peroxide
267 [15]. And this study also showed that 300 μM melatonin could obviously alleviate the
268 drought stress effect on seed germination of wheat. This was in accordance with that
269 of Cui and others [21], reporting that melatonin significantly affected the expression
270 of glycolytic proteins, modulated electron transport in the respiratory chain, and
271 improved energy production.

272 Seed germination is an important biological and dynamic process with
273 mobilization of the major storage reserves. Under water stress, proline content in
274 wheat seed significantly increased [22]. Proline as organic substances can regulate the
275 plasma osmotic potential, and protect the enzymes and plasma membranes under
276 water stress condition [23]. A low concentration of melatonin promoted the synthesis
277 of glycine and succinyl-CoA to influence porphyrins synthesis; high concentration of
278 melatonin lead to proline and carbohydrate synthesis for osmoregulation [20].
279 Melatonin could also accelerate the metabolic flow from the precursor amino acids
280 arginine and methionine to polyamines, which mitigate salt stress on wheat seedling

281 [24]. These suggested that melatonin had directly and indirectly influenced on amino
282 acid content. In this study, Pro and Glu content increased in wheat seed under PEG
283 stress. Low concentration of melatonin (1 and 10 μ M) improved the increasing range
284 of Pro, Glu and Gly content in seed of JM22 under water stress, which was partially
285 conformed to Sarropoulou et al. [20]. However, melatonin decreased the increasing
286 range of Pro and Glu content in seed of HG35. Results showed different
287 drought-resisting ability of two wheat varieties.

288 During germination, amino acids stored in wheat seeds as storage protein are
289 decomposed by hydrolysis [25]. Under abiotic stress, such as salt and water
290 deficiency, free amino acids would play regulation roles as the most hydrophilic and
291 somatically active compounds [26]. Hartmann et al. [27] reported a
292 pathogen-inducible Lys catabolic pathway in plant that generated N-hydroxypipicolinic
293 acid as a critical regulator of systemic acquired resistance to pathogen infection. In
294 this study, Asp, Glu, Phe, and Lys contents were all correlated significantly to one or
295 more indexes of wheat germination. Remarkably, Lys content was significantly and
296 positively correlated with germination percentage, germination index, germination
297 potential, radicle length, and plumule length. A new report showed that histone
298 deacetylase 14, playing a significant role in deacetylation of lysine on histone, was
299 involved in melatonin biosynthesis of *Arabidopsis thaliana* [28]. And future studies
300 should be focused on the interaction between melatonin and prominent amino acid
301 such as lysine changing in different wheat varieties seeds under water stress.

302 **Conclusion**

303 In summary, our data revealed that exogenous melatonin with different concentrations
304 had different effects on germination morphological indexes of winter wheat. 300 μM
305 melatonin could obviously alleviate the adverse effect of drought stress on
306 germination of wheat seed. This was accompanied by an increase in lysine content,
307 which was significantly and positively correlated to seed germination indexes. This
308 suggested Lys may play an important role in regulating drought resistance of wheat
309 seed treated by melatonin. Also, there was genotypic difference in drought resistance
310 due to difference in amino acid content and changing amplitude during wheat
311 germination.

312 **Supporting Information**

313 S1 Table. The objective value corresponding to Fig 3.

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326

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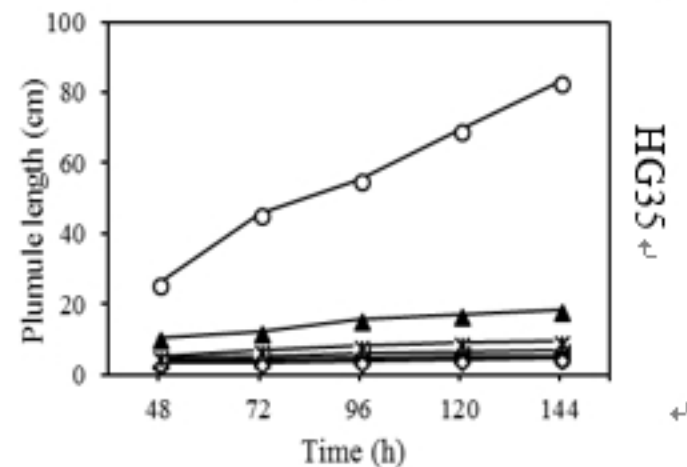
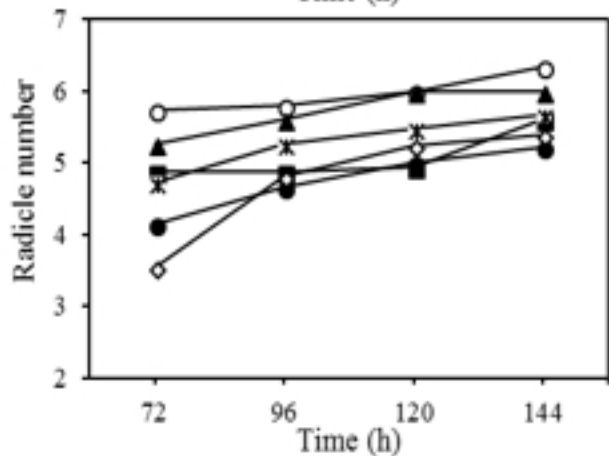
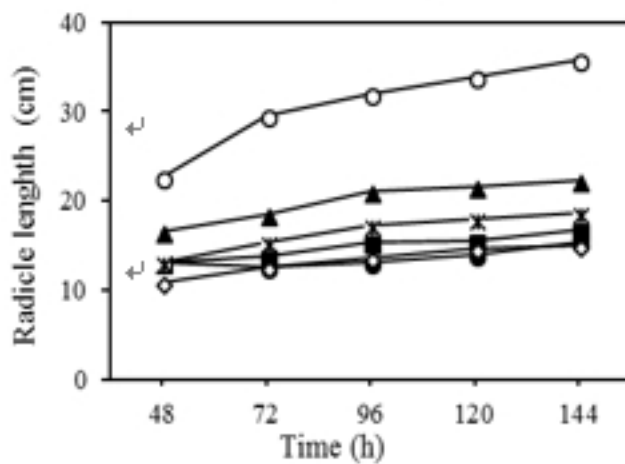
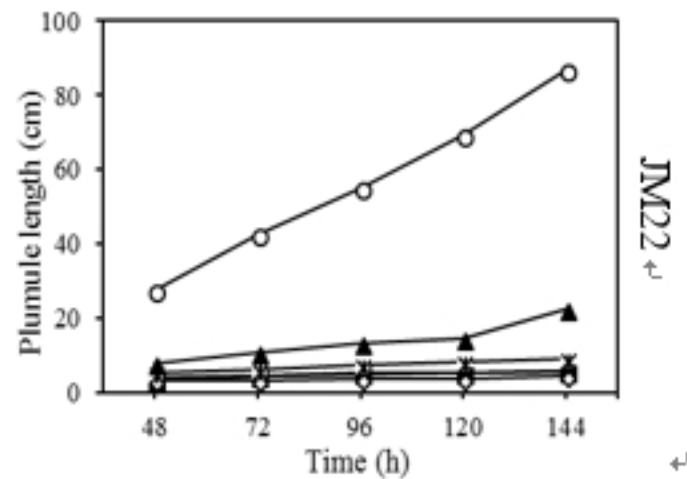
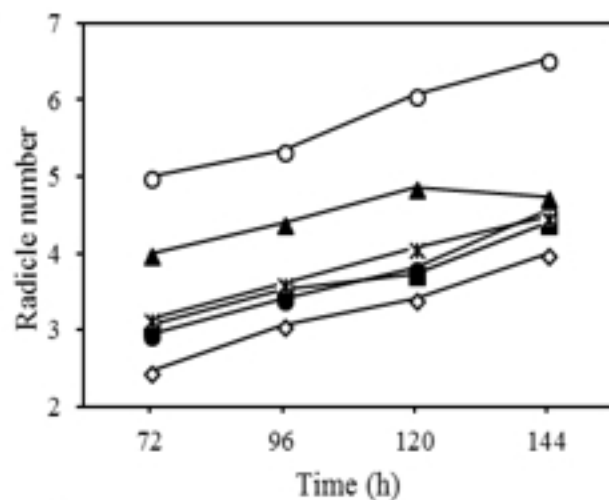
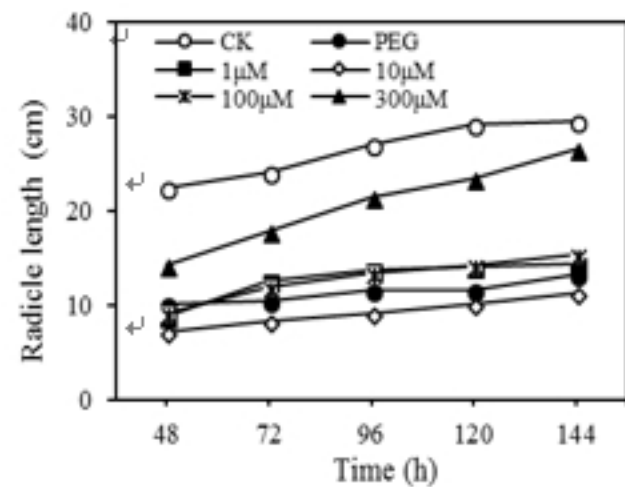


Figure 1

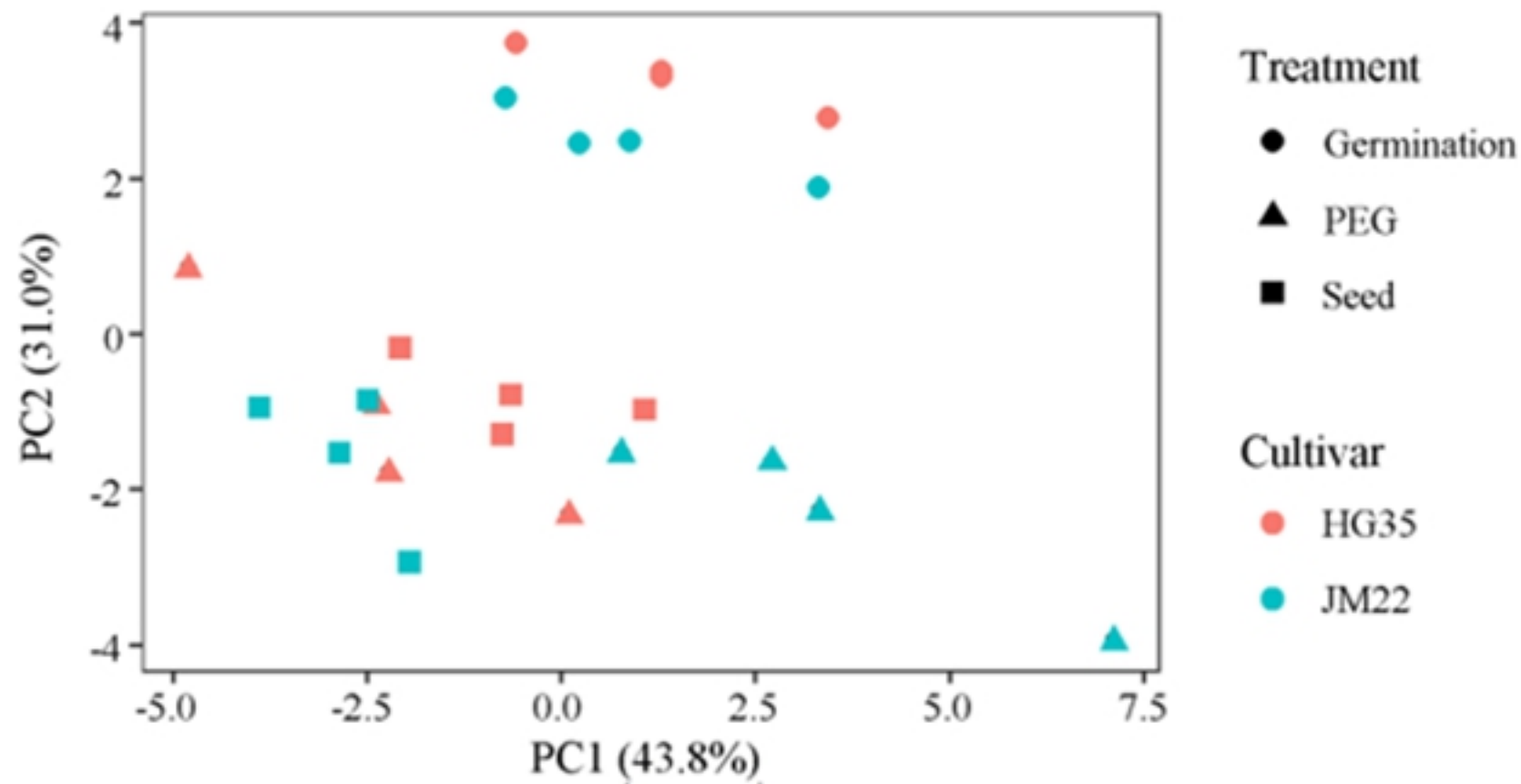


Figure2

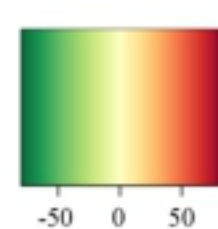


Figure3