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Efficacy of epsilon-poly-L-lysine inhibition of postharvest blue mold in apples and potential mechanisms

Yong Dou[a,b], Michael N Routledge[a,c], Yunyun Gong[a,d], Esa Abiso Godana[a], Solairaj Dhanasekaran[a], Qiya Yang[a], Xiaoyun Zhang[a], Hongyin Zhang[a,*]

[a]School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, Jiangsu, People's Republic of China.

[b]Department of Grain Engineering and Food Drug, Jiangsu Vocational College of Finance & Economics, Huai'an 223003, Jiangsu, People's Republic of China.

[c]Leeds Institute of Cardiovascular and Metabolic Medicine, School of Medicine, University of Leeds, LS2 9JT, UK.

[d]School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK.

***Corresponding author.**

Email address: zhanghongyin126@126.com

Tel.: +86-511-88790211; Fax: +86-511-88780201.

Abstract: *Penicillium expansum* is a major postharvest fungal pathogen and is the causal agent of blue mold decay in apples. Epsilon-poly-L-lysine (ε-PL) is a naturally-occurring polypeptide that has strong antimicrobial activity. It is primarily used to suppress foodborne pathogens in bread, beverage, meat products, etc. The potential application of ε-PL in the management of fungal postharvest diseases of fruit, however, has not been explored. In the present study, the inhibitory effect of ε-PL against blue mold (*P. expansum*) disease of apples and its potential mechanism of action were investigated. Results indicated that 600 mg L^{-1} of ε-PL could effectively inhibit mycelial growth of *P. expansum* in apples. Concentrations of ε-PL > 200 mg L^{-1} also inhibited germination of conidia and germ tube growth of *P. expansum* in potato dextrose broth (PDB). The inhibitory effect increased with increasing concentrations of ε-PL concentration. Further studies indicated that the possible mechanisms involved of ε-PL inhibition of *P. expansum* included the activation of defense-related enzyme activity and gene expression in apple fruit tissues. This included polyphenol oxidase (PPO), catalase (CAT), peroxidase (POD) and phenylalanine ammonialyse (PAL). ε-PL stimulated the production of intracellular reactive oxygen species (ROS) and degraded the integrity of the cell wall and plasma membrane of conidia, resulting in the death of conidial spores of *P. expansum* or their germination.

Key Words: Apple; Blue mold; Induced resistance; Epsilon-poly-L-lysine; Membrane integrity; ROS

1. Introduction

Postharvest infection of fruit by decay fungi is a global problem that results in a significant amount of economic losses annually (Zhang, et al., 2017). Blue mold disease,

38 caused by *P. expansum*, is a major postharvest disease of apple (Errampalli, 2014). Chemical
39 fungicides are conventionally used to inhibit postharvest fungal diseases of apples, but
40 long-term use of chemical fungicides may lead to fungicide resistance in pathogens and
41 environmental pollution (Salomao, et al., 2008; Sansone et al., 2005; Sharma et al., 2009;
42 Förster et al., 2007). Hence, it is important to develop effective, non-toxic, and
43 environmentally-friendly alternatives to manage postharvest apple diseases.

44 ε-PL is a polypeptide consisting of 25-35 lysine residues linked by peptide bonds,
45 formed by the dehydroxylation of the carboxyl group on the α-carbon atom of one lysine and
46 the dehydrogenation of the amino group on the ε-carbon atom of another lysine (Pandey &
47 Kumar, 2014; Shima & Sakai, 1977). The degree of antimicrobial activity of ε-PL is related
48 to its molecular weight (MW). Notably, ε-PL with a MW of 3600 to 4300 Da can
49 significantly inhibit the growth of microorganisms, while ε-PLs with a MW lower than 1300
50 Da do not exhibit antimicrobial activity (Tuersuntuoheti et al., 2019). As a natural
51 preservative originally discovered in *Streptomyces albulus*, synthesized ε-PL is safer than
52 other chemical antimicrobials because it can be completely decomposed into lysine in the
53 human body, an essential amino acid in humans (Bhattacharya et al., 2018; Li et al., 2014ab).
54 No histopathological or carcinogenic effects were observed in rats when a high concentration
55 of ε-PL (20 g kg^{-1}) added to their diet (Hosomi et al., 2015). In fact, many countries,
56 including Japan, the USA, and South Korea currently allow the use of ε-PL as a food
57 preservative based on its antimicrobial efficacy, stability, and safety (Ye et al., 2013; Zhang et
58 al., 2015).

59 The inhibitory effect of ε-PL on foodborne pathogenic bacteria has been widely studied.
60 ε-PL effectively inhibits gram-positive microbes such as *Bacillus subtilis*, *Micrococcus luteus*,
61 and *Staphylococcus aureus*, and even more effectively inhibits gram-negative microbes such
62 as *Salmonella typhymurium*, *Pseudomonas* spp. and *Escherichia coli* O157:H7 (Zhang et al.,
63 2018; Hyldgaard et al., 2014). Previous studies have reported that ε-PL can inhibit
64 postharvest diseases of jujube, citrus, tomato, and kiwifruit (Li et al., 2019; Liu et al., 2017;
65 Sun et al., 2018; Li et al., 2017b). No studies are available, however, on the ability of ε-PL to
66 control postharvest blue mold disease in apples. The purpose of the present study was to
67 determine the ability of ε-PL to inhibit blue mold disease in apples and explore the potential
68 mechanism of action of ε-PL against *P. expansum* both *in vitro* and *in vivo*. Results of the
69 study contribute to sustainable agricultural production and the goal of replacing chemical
70 fungicides with organic compounds for the control and prevention of postharvest diseases of
71 perishable fruit commodities.

72 **2. Material and methods**

73 *2.1. Pathogen*

74 *P. expansum* was isolated from decayed apple tissue, and cultured as described by
75 Zheng et al. (2017). *P. expansum* conidia were collected and preserved in an ultra-freezer at
76 -80 °C. Prior to use in the present study, the pathogen was activated by culturing spores in
77 PDB medium at 25 °C and 150 rpm for 24 h. The conidial suspension was subsequently
78 spread on potato dextrose agar (PDA) and incubated at 25 °C for 7 d. After 7 d of incubation,
79 a *P. expansum* spore suspension was prepared by adding sterile physiological saline to the
80 plate and gently scraping conidia from the culture surface with the use of a sterile pipette

81 head. A hemocytometer was used to determine the concentration of the spore suspension and
82 sterile normal saline was used to dilute the spore suspension to the required concentration.

83 *2.2. Apple fruit*

84 Mature apples (*Malus* × *domestica* Borkh, cv. Fuji) that had not received any chemical
85 treatments were harvested from a fruit orchard in Yantai, Shandong province, China. Apples
86 selected for use in the experiments were uniform in maturity and size, and free of any
87 mechanical or pest damage. Apples were refrigerated at 4 °C immediately after picking.
88 Apples were soaked in 0.1 % (v/v) sodium hypochlorite for 2 min before each experiment to
89 decrease or eliminate surface microbes, rinsed with sterile water, and air-dried (Mahunu et al.,
90 2018).

91 *2.3. ε-PL inhibition of blue mold in apples*

92 The effect of ε-PL on postharvest blue mold of apples was assessed as described by Li et
93 al. (2019) and Ge et al. (2018). Selected apples were administered small wounds at the
94 equator of each apple. Four wounds (5 mm diameter × 3 mm depth) were made with a
95 sterilized cork borer at room temperature. Thirty μL of various concentrations (100, 200, 400,
96 600, or 800 mg L^{-1}) of ε-PL were administered into each wound. Sterilized saline solution
97 was used as the control. The apples were placed in plastic containers previously sterilized
98 with ethanol, wrapped in polyethylene film and placed in a constant temperature chamber at
99 20 °C at 95 % relative humidity (RH). After 24 h, 30 μL of a conidial suspension (1×10^5
100 spores mL^{-1}) of *P. expansum* was pipetted into each wound. The apples were then returned to
101 the same storage conditions for six days. Decay incidence and lesion diameter were evaluated
102 every 24 h. Each treatment consisted of three replications and each replication contained six

103 apples and four wounds per apple. The percentage of decay incidence for each replicate was
104 calculated as follows: number of decayed wounds/total number of wounds × 100. The lesion
105 diameter for each replicate was calculated by measuring the average diameter of all the rotten
106 wounds. The whole experiment was carried out twice.

107 *2.4. Effect of ε-PL on P. expansum mycelial growth in apples*

108 Thirty μL of 600 mg L^{-1} ε-PL was administered in wounds of apple as previously
109 described, with sterilized saline solution used as a control, to study the effect of ε-PL on
110 mycelial growth in apple tissues. Thirty μL of a conidial suspension (1×10^5 spores mL^{-1})
111 was subsequently added into each wound after the apples were stored at 20 °C and 95 % RH
112 for 24 h. The treated apples were then packed as previously described and returned to storage
113 conditions for up to 18 h. Tissue samples were collected from the apple wounds at 9 h, 12 h,
114 and 18 h and prepared for scanning electron microscopy (SEM). The wounded tissue samples
115 were cut into thin slices approximately 1 mm thick with a sterile razor blade and then placed
116 in 2.5 % glutaraldehyde solution at 4 °C for more than 4 h. Samples were then rinsed with 0.1
117 M phosphate buffer system (PBS) pH 7.2 for 1 min. The apple tissue slices were then passed
118 through a series of increasing concentrations of ethanol (30 %, 50 %, 70 %, 90 %), allowing
119 them to stay in each solution for at least 5 min (Pietrysiak et al., 2018; Daniel et al., 2015).
120 Samples were then immediately vacuum freeze-dried for 24 h, and attached to a conductive
121 metal grid, coated with gold/palladium and viewed on a field emission scanning electron
122 microscope (JSM-7001F, JEOL, Japan).

123 *2.5. Effect of ε-PL on the morphology of P. expansum mycelia in vitro*

124 Morphological alterations in the mycelia of *P. expansum* were examined using SEM as
125 described by He et al. (2011). Samples of 1×10^5 spores mL^{-1} were cultured for 9 h at 25 °C
126 and 150 rpm in PDB media amended with ε-PL (0, 200, 400, or 600 mg L^{-1}). Cultures were
127 then centrifuged for 3 min at 8000 × *g* and the precipitates were collected and rinsed with
128 PBS. The collected mycelia were then fixed in 2.5 % glutaraldehyde and dehydrated in
129 ethanol as previously describe. Mycelial samples were then placed on a rectangular cover
130 glass of size 3 mm × 6 mm, and the cover glass was attached to a conductive grid pasted with
131 conductive adhesive. The samples were then dried for 24 h using a vacuum freeze-dryer and
132 coated with gold/palladium. Morphological alterations in the mycelia caused by exposure to
133 the ε-PL were observed using SEM.

134 *2.6. Intracellular ROS determination and plasma membrane integrity*

135 Intracellular ROS and plasma membrane integrity of *P. expansum* spores were examined
136 as previously described (Su et al., 2018; Li et al., 2017a; Shi et al., 2012). Briefly, conidia of
137 *P. expansum* were cultured in PDB media amended with various concentration of ε-PL (0,
138 200, and 400 mg L^{-1}) for 9 h at a constant temperature of 25 °C on a rotary shaker set at 150
139 rpm. The conidial cultures were then centrifuged for 3 min at 8000 × *g* at room temperature.
140 The conidia were washed twice with PBS. The centrifuged samples of conidia were then
141 stained with 10 μM of 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA), and 10 mg L^{-1}
142 propidium iodide (PI) solutions. Samples were placed in a shaking incubator at 30 °C and
143 150 rpm for 30 min in dark. The conidia and germ tubes were then immediately observed
144 under a fluorescence microscope. Three fields of view with about 100 conidia were randomly

145 selected to take pictures, and the ratio of the number of conidia producing ROS and the
146 number of conidia with cell leakage to the total number of conidia was calculated.

147 *2.7. Examination of cellular leakage in P. expansum*

148 Leakage of cellular contents from *P. expansum* mycelia was examined as described by
149 Cai et al. (2015), Cui et al. (2018), and Zhu et al. (2019). *P. expansum* spores were on PDA
150 media for 7 d. Conidia were then collected from the PDA plate and spore concentration was
151 adjusted to 1×10^5 spores mL^{-1} with sterile saline solution and the aid of a hemocytometer.
152 The collected conidia were cultured in PDB medium on a shaking incubator at 150 rpm and
153 25 °C for 72 h. Samples of the cultures were then collected and washed with sterile distilled
154 water. The washed mycelia were resuspended in 100 mL of various concentrations ε-PL (0,
155 200, and 400 mg L^{-1}), and cultured at 25 °C for 1, 2, 3, and 4 h on a rotary shaker set at 150
156 rpm. The mycelial suspensions were then centrifuged for 3 min at 8000 × *g*. The resulting
157 supernatants were used to assay the level of soluble proteins and nucleic acids which would
158 be indicative of cell leakage. The Bradford assay (Bradford, 1976) was used to quantify
159 soluble proteins. The concentration of nucleic acids was determined by measuring
160 absorbance at 260 nm (A_{260nm}). Three replicates were used for each treatment and the
161 experiment was repeated twice.

162 *2.8. Analysis of enzyme activity and corresponding gene expression in apples*

163 *2.8.1. Defense-related enzyme activity*

164 Apple samples were surface sterilized and artificially wounded as previously described.
165 Thirty μL of 600 mg L^{-1} ε-PL was then administered into each wound, with sterile saline
166 used as a control. The apples were placed in plastic container wrapped in polyethylene film

167 and stored at 20 °C and 95 % RH. Apple tissues surrounding the wounds were collected and
168 mixed at 0, 1, 2, 3, 4, 5, 6, and 7 d after treatment. The mixed tissue samples were divided
169 into two parts, one portion of the samples was immediately frozen in liquid nitrogen and kept
170 at -80 °C for subsequent determination of defense-related gene expression, and the other
171 portion was used to assay defense-related enzyme activity. Each treatment consisted of three
172 replications and each replication contained six apples and four wounds per apple.

173 Tissue samples (about 2 g) and a little silica sand were placed together in a pre-frozen
174 mortar and ground in 1 mL precooled PBS (50 mM, pH 7.8) with 0.038 % ethylene
175 diaminetetraacetic acid and 1 % polyvinylpyrrolidone. The ground apple tissue samples were
176 then transferred to a centrifuge tube and washed by adding 9 mL of PBS, and centrifuged at
177 12000 × *g* for 8 min at 4 °C (Zhao et al., 2020). The enzyme activity of PAL, POD, PPO, and
178 CAT in the collected supernatant was determined following the protocols described in
179 Apaliya et al. (2017), Wang et al. (2019), and Wang et al. (2018). All the apple tissue weight
180 mentioned in this paper refer to the fresh weight. Enzyme activity is indicated as U g^{-1} fresh
181 weight of samples.

182 PAL activity was determined as described by Wang et al. (2019) with some
183 modifications. Crude enzyme extract (1mL) was added into 3 mL, 50 mM borate buffer
184 solution (pH 8.8, preheated at 37 °C for 5 min) containing 10 mM phenylalanine. The initial
185 absorbance value was measured at 290 nm and the test time was accurately recorded. One mL
186 of 50 mM PBS (pH 7.8) was used as the blank. The absorbance values of the samples and
187 control were measured again after 1 h at 290 nm. PAL enzyme activity was calculated by

188 subtracting the two absorbance values. One unit of PAL activity was defined as an increase of
189 0.01 absorbance unit per hour at 290 nm.

190 POD activity was determined according to Apaliya et al. (2017) with some
191 modifications. Crude enzyme extract (0.2 mL) was added into 2.2 mL, 50 mM PBS (pH 6.4,
192 containing 0.3 % guaiacol). Then 0.6 mL of 0.3 % H_2O_2 (50 mM PBS, pH 6.4) was added
193 and samples were incubated at 30 °C for 6 min. POD activity was determined by measuring
194 absorbance at 470 nm every 60 s for 3 min. The blank control consisted of 0.2 mL 50 mM
195 PBS (pH 6.4). POD activity was determined by and increase of 0.01 absorbance unit per
196 minute at 470 nm.

197 PPO activity was measured according to the method previously described by Wang et al.
198 (2018) with some modifications. The reaction mixture was composed of 0.2 mL crude
199 enzyme extract and 2.8 mL pyrocatechol of 0.1 mM (50 mM PBS, pH 6.4) and was incubated
200 at 30 °C for 6 min. POD activity was determined by measuring absorbance at 398 nm every
201 60 s for 3 min. The blank control was the same one used in the POD assay. PPO activity was
202 calculated based on an increase of 0.01 absorbance unit per minute at 398 nm.

203 CAT activity was determined according to Wang et al. (2019) with some modifications.
204 Crude enzyme extract (0.2 mL) was added into 2.8 mL H_2O_2 of 30 mM (50 mM PBS, pH
205 7.8). Absorbance was recorded 240 nm every 60 s for 3 min. The blank control was the same
206 one used in the POD assay. One unit of CAT activity was defined as a decrease of 0.01
207 absorbance unit per minute at 240 nm.

208 *2.8.2. Reverse transcription - quantitative polymerase chain reaction (RT-qPCR) analysis of*
209 *defense-related gene expression*

210 Frozen apple tissue (2 g) stored at -80 °C was used to extract RNA with a plant total
211 RNA purification kit (Sangon Biotech, Shanghai, China) according to the manufacturer's
212 instructions. Absorbance at 260 and 280 nm measured in a spectrophotometer was used to
213 evaluate the purity and quantity of RNA. The integrity and content of RNA was determined
214 using and RNA Nano 6000 assay kit and a Bioanalyzer 2100 system (Agilent Technologies,
215 CA, USA). First strand cDNA was synthesized from total RNA using a PrimeScript RT
216 reagent kit and gDNA Eraser (Takara-Dalian, China). Specific primers for the evaluated
217 genes were designed with Primer Premier 5.0 software (PREMIER Biosoft International,
218 Palo Alto, CA, USA) and listed in Table 1. RT-qPCR was performed with a Biorad CFX96
219 Real Time PCR System (Applied Biosystems, USA). The PCR conditions were the same as
220 those described in Wang et al. (2019). Amplification products have tested the specificity
221 in accordance with the melting curve analysis was used to evaluate the specificity of the
222 primers used to generate the amplification products. RT-qPCR was carried out using TB
223 Green® Fast qPCR Mix (TAKARA BIO Inc., Shiga, Japan) in an ABI PRISM 7500
224 Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's
225 instructions. The amplification protocol was 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s,
226 and 62 °C for 15 s. Relative gene expression levels was determined using $2^{-\Delta\Delta CT}$ method
227 using the Ct value of apple *Actin* for normalization (Livak and Schmittgen, 2001). The
228 RT-qPCR analysis was conducted for three biological and technical replicates.

229 *2.9. Statistical analysis of data*

230 Each treatment consisted of three biological replicates and all the experiments were
231 repeated at least twice. Analysis of variance (ANOVA) was carried out using SPSS 22.0

232 software (SPSS Inc., Chicago, IL, USA). Tukey's test was used for means separations, and P
233 < 0.05 was considered as statistically significant.

234 **3. Results**

235 *3.1. Inhibitory effect of ε-PL on blue mold in apples*

236 Inhibitory effect of ε-PL on blue mold infection of apples varied with the concentration
237 of ε-PL (Fig. 1). Decay incidence and lesion diameter in the 100 mg L^{-1} ε-PL treatment was
238 not significantly different from the control, indicating that lower concentrations of ε-PL were
239 ineffective against the growth of blue mold in apples. In contrast, decay incidence and lesion
240 diameter decreased significantly with the use of ε-PL concentration ranging from 200 to 800
241 mg L^{-1}. Notably, a major decrease was observed in the 600 mg L^{-1} treatment group. While
242 results indicated that 600 mg L^{-1} ε-PL exhibited the greatest ability to inhibit blue mold, use
243 of a ε-PL concentration of 800 mg L^{-1}, decay incidence and decay diameter significantly
244 increased above the 600 mg L^{-1} ε-PL. This suggests that highest concentration of ε-PL may
245 have been cytotoxic to apples.

246 *3.2. Effect of ε-PL on P. expansum mycelial growth in vivo*

247 As shown in Fig. 2, SEM observations revealed that ε-PL significantly inhibited the
248 growth of *P. expansum* in apple tissues. The conidia of *P. expansum* in the wounds of the
249 control group began to germinate after 12 h, and elongated germ tubes were clearly evident.
250 Later observations of the control group indicated that the fungus readily grew in the wounded
251 tissues as evidenced by the copious amounts of visible mycelia. In contrast, germination of
252 conidia in the ε-PL-treated wounds was not observed until at least 18 h after inoculation, and
253 the surface of condia appeared shriveled and shrunken. These results indicate that the conidia

254 in the ε-PL-treated wounds were under stress and may have been subject to a loss of cellular
255 integrity, thus resulting in an inhibition of mycelial growth.

256 *3.3. Effect of ε-PL on the morphology of P. expansum mycelia in vitro*

257 The morphology of mycelia and germ tubes of *P. expansum* treated with ε-PL were
258 observed using SEM. *P. expansum* conidia that were not treated with ε-PL germinated and
259 developed germ tubes that had a smooth surface (Fig. 3A). Some of the spores treated with
260 200 mg L^{-1} ε-PL developed germ tubes (Fig. 3B), while others failed to germinate. The
261 surface of the germ tubes appeared rough and irregular, and in some cases, it appeared as if
262 the cellular contents had leaked out. At 400 mg L^{-1} (Fig. 3C), only a few spores germinated
263 normally, and most of them lost their morphological integrity. The damaged germ tubes
264 appeared irregular with coarse surfaces. Cellular contents had leaked out and formed
265 aggregates that adhered to the shriveled germ tubes. All of the conidia treated with 600 mg
266 L^{-1} ε-PL failed to germinate and the spores appeared degraded, making them hard to
267 recognize (Fig. 3D). Leaked cellular materials also appeared as aggregates that adhered to the
268 degraded spores. These results indicated that the level of spore degradation increased with
269 increasing concentrations of ε-PL.

270 *3.4. Intracellular ROS and loss of cell membrane integrity*

271 Fig. 4A ROS production in *P. expansum* spores treated with ε-PL was monitored using
272 DCFH-DA staining. The intensity of the green fluorescence of conidia treated with 400 mg
273 L^{-1} ε-PL was stronger than it was in conidia treated with 200 mg L^{-1} ε-PL. The percentage of
274 conidia exhibiting green fluorescence in the 200 mg L^{-1} and 400 mg L^{-1} ε-PL treatment
275 groups was 15.42 % and 71.37 %, respectively (Fig. 4C). These results indicate that the

276 number of spores producing ROS increased as the concentration of ε-PL increased. ε-PL also
277 disrupted the integrity of the plasma membrane in spores of *P. expansum*, as determined using
278 PI staining (Fig. 4B). When the concentration of ε-PL was increased from 200 to 400 mg L^{-1},
279 the corresponding red fluorescence intensity was significantly enhanced (Fig. 4B), and the
280 percentage of conidia that appeared to lose membrane integrity also increased from 10.28 %
281 to 52.36 % (Fig. 4D).

282 *3.5. Effect of ε-PL on the loss of cellular contents in vitro*

283 The leakage of cellular soluble proteins (Fig. 5A) and nucleic acids (Fig. 5B) from the
284 mycelia of *P. expansum* into the culture medium increased dramatically, relative to the control,
285 when the concentration of ε-PL was increased from 200 mg L^{-1} to 400 mg L^{-1}. The mycelia of
286 *P. expansum* began to release soluble proteins and nucleic acids after one hour of exposure to
287 ε-PL. The level of leakage of soluble proteins and nucleic acids into the culture medium
288 reached its highest level, after 4 h, indicating that most of the mycelia had been disrupted by
289 this time. The amount of materials leaked into the culture medium was positively correlated
290 with the concentration of ε-PL. These results indicate that ε-PL damaged the integrity of the
291 plasma membrane of *P. expansum* mycelia resulting in the loss of cellular contents into the
292 culture medium.

293 *3.6. Defense-related enzyme activity and corresponding defense-related gene expression l in*
294 *apples*

295 *3.6.1. Effect of ε-PL on defense-related enzyme activity in apple fruit*

296 The pattern of PAL activity in treated and untreated apple tissue is presented in Fig. 6A.
297 While the overall pattern was similar in both ε-PL-treated and control apple tissues, distinct

298 differences were also evident. Over the course of seven days after being treated with ε-PL,
299 PAL enzyme activity first rose and then fell, and then subsequently rose and fell once more.
300 PAL enzyme activity in the treatment group was similar to the control group on day 3 but
301 otherwise significantly higher than the control group on the other days. PAL activity reached
302 its maximum on day 4 in both treatment and control groups of apples.

303 The pattern of PPO activity in both ε-PL-treated and control apple tissues was similar to
304 the results obtained for PAL (Fig. 6B). PPO activity increased to a maximum peak after 4 d.
305 PPO activity was always higher in ε-PL-treated apple tissues than in non-treated, control
306 apple tissues. As indicated in Fig. 6C and 6D, the pattern of CAT and POD activity in
307 ε-PL-treated and control apple tissues apples was also similar to the results obtained for PAL
308 and PPO. CAT and POD exhibited their highest activity in both groups on day 4 and 5,
309 respectively. Notably, the level of CAT and POD activity was always higher in ε-PL-treated
310 apple tissues than it was in the non-treated tissues. These studies suggest that wounding
311 induces defense-related enzyme activity but that treatment of wounds with 600 mg L^{-1} ε-PL
312 induces a higher level of enzyme activity, which presumably contributed to the inhibition of
313 blue mold (*P. expansum*).

314 *3.6.2. Effect of ε-PL on defense-related enzyme gene expression in apple fruit*

315 The expression level of the genes encoding the defense-related enzymes that were
316 measured were also assessed in the same samples by RT-qPCR. From, *PAL* expression in
317 apples began to rise one day after being treated with ε-PL, and reached a peak of expression
318 at 4 and 5 days (Fig. 7A). *PAL* expression was about 4.3 times higher in than the control in
319 ε-PL-treated apple tissues on the fifth day after treatment. ε-PL treatment also induced the

320 up-regulation of both *PPO* and *CAT* beginning on day 1, all the way through day 7 (Fig. 7B
321 and Fig. 7C, respectively). The maximum level of *PPO* expression in ε-PL-treated apple
322 tissues was observed on the 2nd and 5th day after treatment, at which time it was 5.45 times
323 higher than in the control. *CAT* expression in ε-PL-treated apple tissues reached its highest
324 value on the 4th day after treatment, at which time it was 4.05 times higher than it was in the
325 control. *POD* expression in ε-PL-treated apple tissues was up-regulated on the first, fifth,
326 sixth, and seventh day after treatment (Fig. 7D). The highest level of *POD* expression in
327 ε-PL-treated apple tissues occurred on the first day, at which time it was 3.44 times higher
328 than the control (Fig. 7D). In general, the expression level of the measured defense-related
329 enzyme genes was significantly up-regulated in apple tissues by treatment of wounds with
330 600 mg L^{-1} ε-PL. These results were in general agreement with the measured levels of activity
331 of the corresponding enzymes.

332 **4. Discussion**

333 ε-PL is a safe, non-toxic natural food preservative that is widely used to control
334 foodborne pathogenic bacterial contamination in baked goods, cooked meat products, fruit
335 and vegetable beverages, and noodle products. Despite its antimicrobial properties, it has
336 been rarely tested for its ability to inhibit fungal contamination of food products or
337 postharvest diseases of fruit. Previous studies have been conducted on the inhibitory effect of
338 ε-PL on citrus fruit disease caused by *Pencillium digitatum* and jujube fruit disease caused by
339 *Botrytis cinerea*, however, the ability of ε-PL to induce disease resistance in fruit has not been
340 fully investigated in these studies (Liu et al., 2017; Li et al., 2019). More specifically, studies
341 on the inhibitory effect of ε-PL on blue mold disease in apples or its potential effect on the

342 induction of disease resistance has not been conducted. The present study is the first report on
343 the use of ε-PL to inhibit postharvest decay in apple fruit caused by *P. expansum.* Results of
344 our study revealed that ε-PL significantly inhibited conidial germination in *P. expansum* and
345 mycelial growth *in vitro* and *in vivo*. Furthermore, the study provided evidence of the ability
346 of ε-PL to induce a resistance response in apples.

347 ε-PL in a concentration range of 400 mg L^{-1} and 600 mg L^{-1} provided effective
348 protection against blue mold decay in apples. Concentrations above or below this range were
349 comparatively less efficient in inhibiting blue mold decay. Compared to the antifungal
350 activity *in vitro*, a higher concentration of ε-PL was needed to effectively inhibit germination
351 and mycelial growth in treated apples than was needed to achieve the same effect under *in*
352 *vitro* culture conditions. There are three possible reasons to explain these results. Firstly, ε-PL
353 may be absorbed into the apple tissue after it is administered, thereby reducing the amount of
354 ε-PL to which the fungus is exposed. Secondly, ε-PL entering the apple tissue may react with
355 the apple tissue matrix, changing the molecular structure of the ε-PL, thus reducing its
356 antimicrobial properties. Thirdly, a low concentration of ε-PL may not be sufficient to
357 activate a resistance response in apple tissues, while too high a concentration may be
358 cytotoxic to apple cells which may die or leak nutrients making them available for fungal
359 growth. Notably, our study indicated that the optimal concentration of ε-PL needed to inhibit
360 blue mold decay in apples was significantly lower than the concentration needed to inhibit *P.*
361 *digitatum* in citrus fruit and *B. cinerea* in jujube fruit. This would make the application of
362 ε-PL in apples more economic and also reduce potential residues.

363 DCFH-DA and PI staining methods were used to detect the level of ROS production and
364 quantify the level of damaged cells, respectively. The DCFH-DA results revealed that high
365 levels of ROS were produced and the results with PI revealed that that the number of
366 damaged *P. expansum* spores was very high when they were exposed to 600 mg L^{-1} ε-PL.
367 Excessive ROS production results in oxidative injury to mitochondria and cell components
368 such as proteins and lipids. This damage results in cell dysfunction and the suppression of
369 spore germination, mycelial growth, all of which reduce pathogenicity. SEM observations
370 revealed that the leakage of cell contents from spores was positively proportional to the
371 concentration of ε-PL. This suggests that the higher concentrations of ε-PL degrade the cell
372 walls of spores and cause a loss of membrane integrity, in some cases killing the spores of *P.*
373 *expansum*.

374 PAL, PPO, CAT, and POD are four important defense-related enzymes that contribute to
375 innate disease resistance in fruits. The level of activity of these enzymes are positively
376 correlated with fruit disease resistance, and thus, they can be used as indicators of the level of
377 fruit disease resistance (Qin et al., 2015; Zhang et al., 2016). Phenolic compounds, flavonoids,
378 and lignin are primary antifungal compounds which are synthesized in the phenylpropanoid
379 pathway. PPO has a vital function in host plant defense mechanisms, participates in the
380 metabolism of phenols, converts phenolic compounds to more toxic quinine, and so its
381 activity would enhance disease resistance in apple fruit tissues. PAL is a crucial rate-limiting
382 enzyme in the shikimic acid pathway that plays a primary role in the production of secondary
383 metabolites, including phenolic compounds, and lignin. POD and CAT are antioxidant
384 enzymes that help prevent excessive ROS accumulation in plant cells. In that the present

385 study, PAL, PPO, CAT, and POD activity significantly increased in apple tissues treated with
386 600 mg L^{-1} ε-PL for 24 h, indicating that ε-PL induced a defense response in apple tissues.
387 The expression level of genes encoding the four defense enzymes were assessed by RT-qPCR,
388 and further confirmed that 600 mg L^{-1} ε-PL of activates a defense response in apple tissues.
389 Our results demonstrated that ε-PL induced apples to up-regulate the expression of *PPO*, *CAT*,
390 *POD*, *and PAL* genes, which resulted increased activity of the four corresponding
391 defense-related enzymes, leading to enhanced resistance to blue mold decay in apple fruit.
392 Overall, the increased activity of defense-related enzymes may have contributed to enhanced
393 resistance by increasing the synthesis of antifungal compounds and managing oxidative stress
394 in apple cells. Future experiments will further explore the molecular mechanism underlying
395 the ε-PL-induced enhanced resistance of apple to blue mold decay, and include both
396 transcriptomic and proteomic analyses.

397 **5. Conclusions**

398 Results of the present study demonstrated that ε-PL effectively inhibits postharvest blue
399 mold decay in apples. The optimal concentration of ε-PL in apples was 600 mg L^{-1}.
400 Concentration of ε-PL above 200 mg L^{-1} had an inhibitory effect on *P. expansum in vitro*,
401 with the level of inhibition being concentration dependent. The study also suggested three
402 possible mechanisms associated with ε-PL inhibition of *P. expansum.* Firstly, ε-PL has the
403 ability to induce the up-regulation of defense-related gene expression and increase
404 corresponding defense-related enzyme activity of PAL, PPO, CAT, and POD in apples tissues.
405 Secondly, exposure of *P. expansum* to ε-PL resulted in excessive intracellular ROS
406 production in spores, which may have injured or killed spores, thus reducing or eliminating

407 spore germination or mycelial growth. Thirdly, ε-PL may degrade the cell wall and reduce the
408 integrity of the plasma membrane of *P. expansum* conidia, causing a large amount of cellular
409 materials to leak out of the cells, again reducing or eliminating spore germination and
410 mycelial growth. Further molecular studies will be required, however, to firmly establish the
411 mechanisms by which ε-PL limits spore germination and mycelial growth of *P. expansum*,
412 and enhances disease resistance in apple tissues.

413 **Conflict of interest**

414 The authors declare that there is no conflicts of interest.

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591

592

593 **Table. 1** Primers used in RT-qPCR reactions of defense-related relative gene expressions in

594 apples.

Gene name	Accession number	Primer	Primer Sequence (5' → 3')
Polyphenoloxidase (*PPO*)	LOC103446446	F-*PPO*- qRT	ATGCCAAGTCCAAGAGCCAA
		R-*PPO*- qRT	CCAGTGCCGGATTGGTTGTA
Peroxidase (*POD*)	LOC103444817	F-*POD*- qRT	AAGCCTATAGCCCCACCAGA
		R-*POD*- qRT	CTTGAAGCTACGTGGGTCGT
Catalase (*CAT*)	LOC103445262	F-*CAT*- qRT	AGACACCTGTCATTGTGCGT
		R-*CAT*- qRT	ACACGAGGGTCGGATAGGG
Phenylalanine ammonia lyase (*PAL*)	LOC103419282	F-*PAL*- qRT	GGCATTTGGAGGAGAACTTG
		R-*PAL*- qRT	AGAACCTTGAGGGGTGAAGC
ACTIN	LOC103447714	F-*ACTIN*- qRT	CCCAAAGGCTAATCGGGAGAAA
		R-*ACTIN*- qRT	ACCACTGGCGTAGAGGGAAAGA

595

596 **Figure Caption**

597 **Fig. 1.** The effect of different concentrations of ε-PL on blue mold decay in apples caused by

598 *Penicillium expansum.* Apples were treated with 30 μL of 0, 100, 200, 400, 600, or 800 mg

599 L^{-1} ε-PL for 24 h at 20°C, 95% RH. Subsequently, 30 μL of a *P. expansum* spore suspension

600 (1×10^5 spores mL^{-1}) was administered into each wound. Fruit were then stored for 6 d.

601 Representative photos were taken after 6 days (A), and decay incidence (B) and lesion

602 diameter (C) were assessed on days 3, 4, 5, and 6. Each treatment consisted of three replicates

603 and each replicate contained six apples and four wounds per apple, the experiment was

604 repeated twice. Data presented represent the mean ± standard error, n = 3. Different small

605 letters over the columns indicate significant differences between treatments on the day of

606 assessment as determined by a Duncan's multiple range test ($P < 0.05$).

607

608 **Fig. 2.** Scanning electron microscopy (SEM) images of the effect of ε-PL on germination of *P.*
609 *expansum* conidia in apples. ε-PL (30 μL) at 600 mg L^{-1} and sterilized saline solution (30 μL)
610 as a control was added into the wounds made in apples. Apples were stored at 20 °C and
611 95 % RH for 24 h prior to administering 30 μL of a spore suspension (1×10^5 spores mL^{-1}) of
612 *P. expansum* into each wound, after which the apples were then placed under the same storage
613 conditions. Samples of the wounded tissues were removed from the apple at 9 h, 12 h, and 18
614 hand prepared for SEM. The scale bar represents 10 μm. Each treatment consisted of three
615 replicates and the experiments were repeated twice.
616
617 **Fig. 3.** Scanning electron microscopy (SEM) images of mycelia and conidia of *P. expansum*
618 treated with (A) 0 mg L^{-1} (B) 200 mg L^{-1} (C) 400 mg L^{-1} (D) 600 mg L^{-1} ε-PL *in vitro* for 9 h.
619 *P. expansum* spore suspensions (1×10^5 spores mL^{-1}) were cultured in PDB media amended
620 with ε-PL (0, 200, 400, or 600 mg L^{-1}) at 25 °C on a rotary shaker set at 150 rpm. After 9 h,
621 the cultures were centrifuged for 3 min at 8000 × *g* and prepared for SEM. The scale bar
622 represents 1μm. Each treatment consisted of three replicates and the experiments were
623 repeated twice.
624
625 **Fig. 4.** Effect of ε-PL on reactive oxygen species (ROS) production and membrane integrity
626 of *P. expansum* conidia *in vitro*. Conidia were incubated in different concentrations of ε-PL (0,
627 200, and 400 mg L^{-1}) for 9 h and then stained with 10 μM of 2′,7′-dichlorodihydrofluorescein
628 diacetate (DCFH-DA) or 10 mg L^{-1} of propidium iodide (PI). ROS production in conidia is
629 indicated by green fluorescence (A), while loss of membrane integrity is indicated by red

630 fluorescence (B). Percentage of conidia exhibiting high levels of ROS (C), and the percentage
631 of conidia exhibiting a loss of membrane integrity (D). Three fields of view, each containing
632 at least 100 conidia were randomly selected for photographing. Each treatment consisted of
633 three replicates and the experiments were repeated twice. Scale bar represents 50 μm. Data
634 represent the mean ± standard error of the mean, n = 3. Different small letters over columns
635 indicate significant differences between treatments as determined by a Duncan's multiple
636 range test ($P < 0.05$).

637

638 **Fig. 5.** Effect of different concentrations of ε-PL on the amount of soluble protein and nucleic
639 acid leakage from *P. expansum* conidia treated with 0, 200, and 400 mg L^{-1} ε-PL. The washed
640 mycelia were resuspended in 100 mL of sterile distilled water containing ε-PL and placed at
641 25 °C on a rotary shaker set at 150 rpm. The leakage of soluble proteins (A) is expressed as
642 milligrams per liter of solution, while leakage of nucleic acids (B) is expressed as absorbance
643 value at 260 nm (A_{260nm}). Treatments: CK (sterile distilled water), A (ε-PL at 200 mg L^{-1}), B
644 (ε-PL at 400 mg L^{-1}). Each treatment contained three replicates, and the experiment was
645 repeated twice. Data presented represent the mean ± standard error of the mean, n = 3.
646 Asterisk (*) indicates significant differences between the control and treatment by a Duncan's
647 multiple range test ($P < 0.05$).

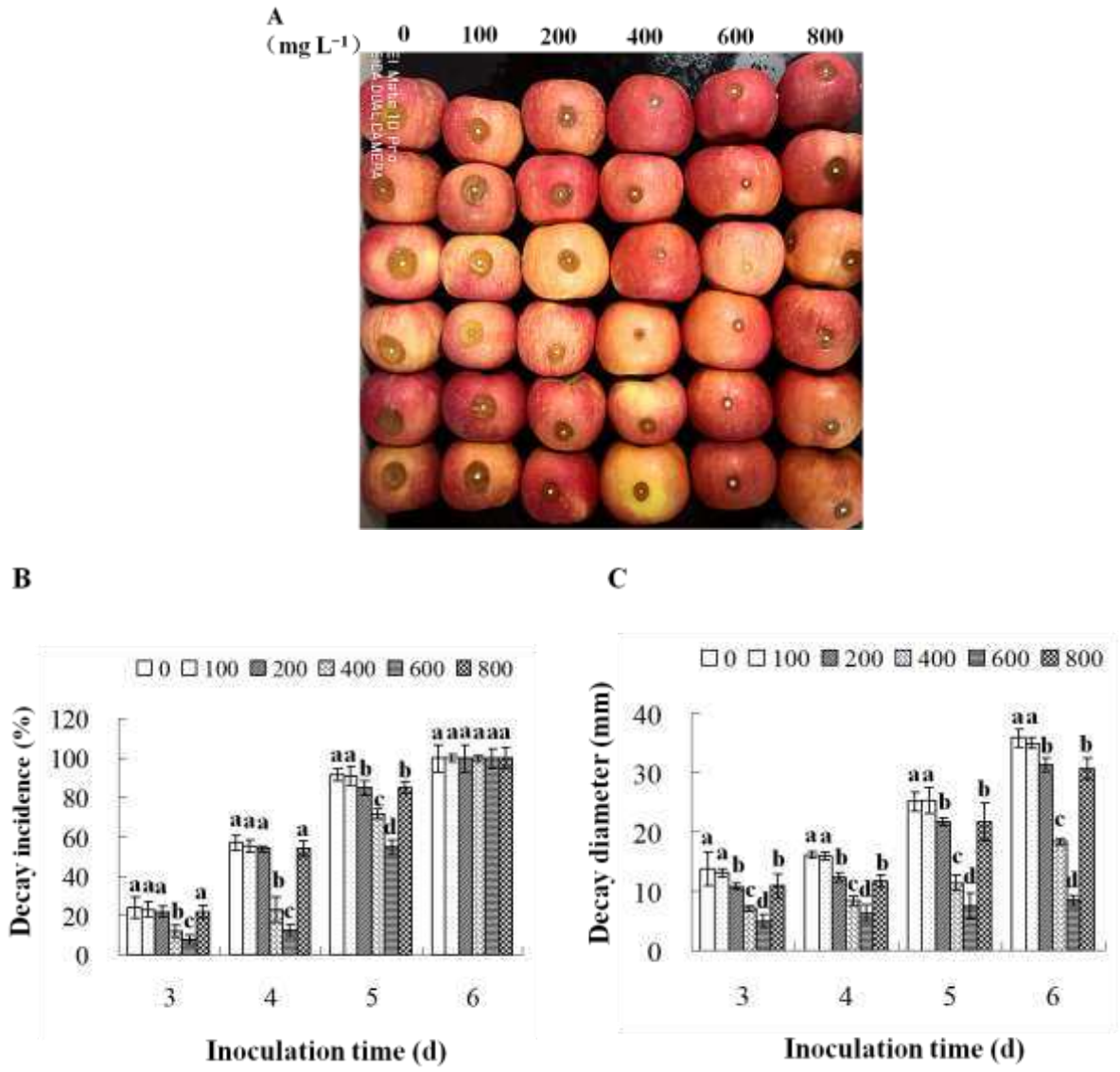
648

649 **Fig. 6.** Time course changes in defense-related enzyme activity in apples induced by ε-PL.
650 Each wound in apples were administered 30 μL of ε-PL, while 30 μL of sterilized saline
651 solution was used as a control. Apple tissue around wounds was collected at 0, 1, 2, 3, 4, 5, 6,

652 and 7 d after treatment and assayed for (A) PAL, (B) PPO, (C) CAT, and (D) POD activity.
653 Treatments: CK - saline control, and ε-PL at 600 mg L^{-1}. Each treatment consisted of three
654 replicates and the experiment was repeated twice. Data represent the mean ± standard error ,
655 n = 3. Asterisk (*) indicates significant differences between the control and treatment by a
656 Duncan's multiple range test ($P < 0.05$).
657
658 **Fig. 7.** Time course changes in defense-related enzyme gene expression in apples treated with
659 ε-PL. Each wound in apples were administered 30 μL of ε-PL, while 30 μL of sterilized
660 saline solution was used as a control. Apple tissue around wounds was collected at 0, 1, 2, 3,
661 4, 5, 6, and 7 d after treatment and the tissues were used to assess defense-related gene
662 expression of (A) *PAL*, (B) *PPO*, (C) *CAT* and (D) *POD*. Treatments: CK - the saline control,
663 and ε-PL at 600 mg L^{-1}. Each treatment consisted of three replicates and the experiment was
664 repeated twice. Data represent the mean ± standard error, n = 3. Different small letters above
665 columns indicate significant differences between the level of expression in the apple tissues
666 treated with ε-PL as determined by a Duncan's multiple range test ($P < 0.05$).
667

668 **Fig.1**

669



670

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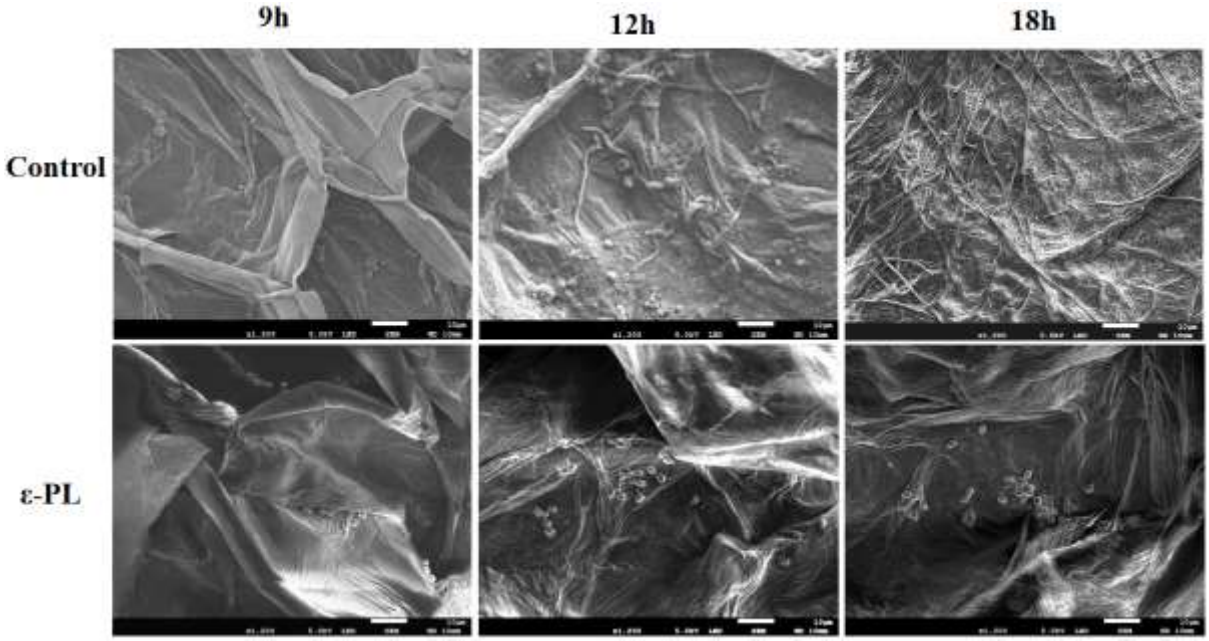
672

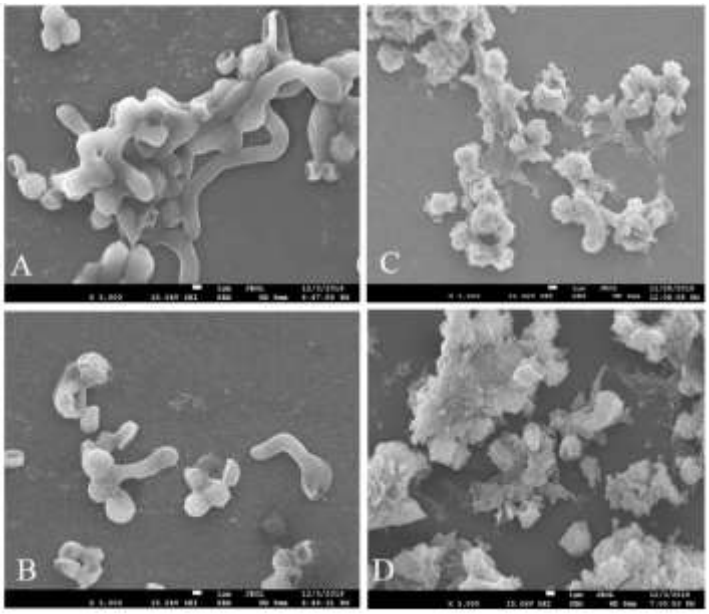
673 **Fig.2**



674

675

676 **Fig.3**



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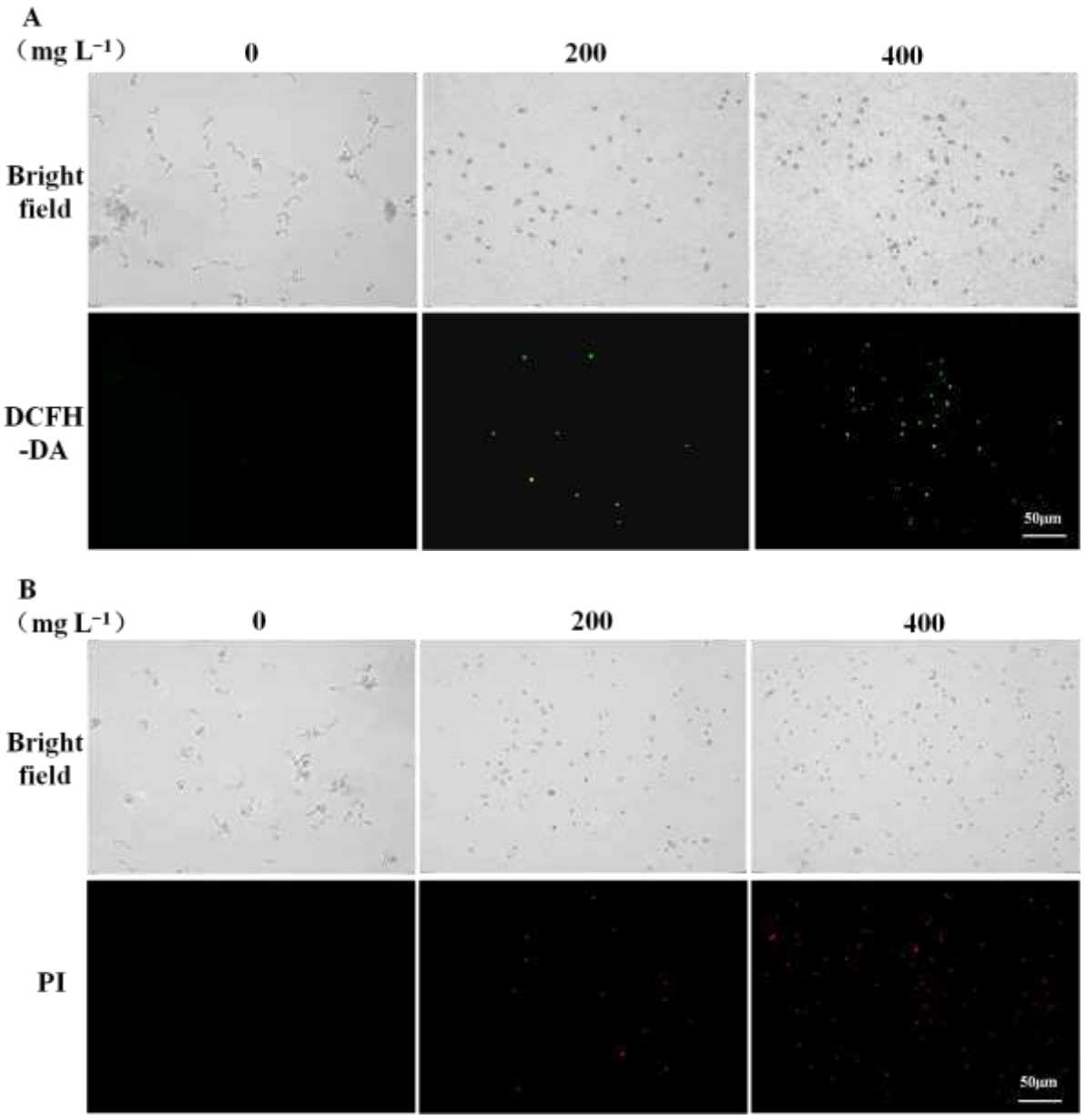
679

680 **Fig.4**



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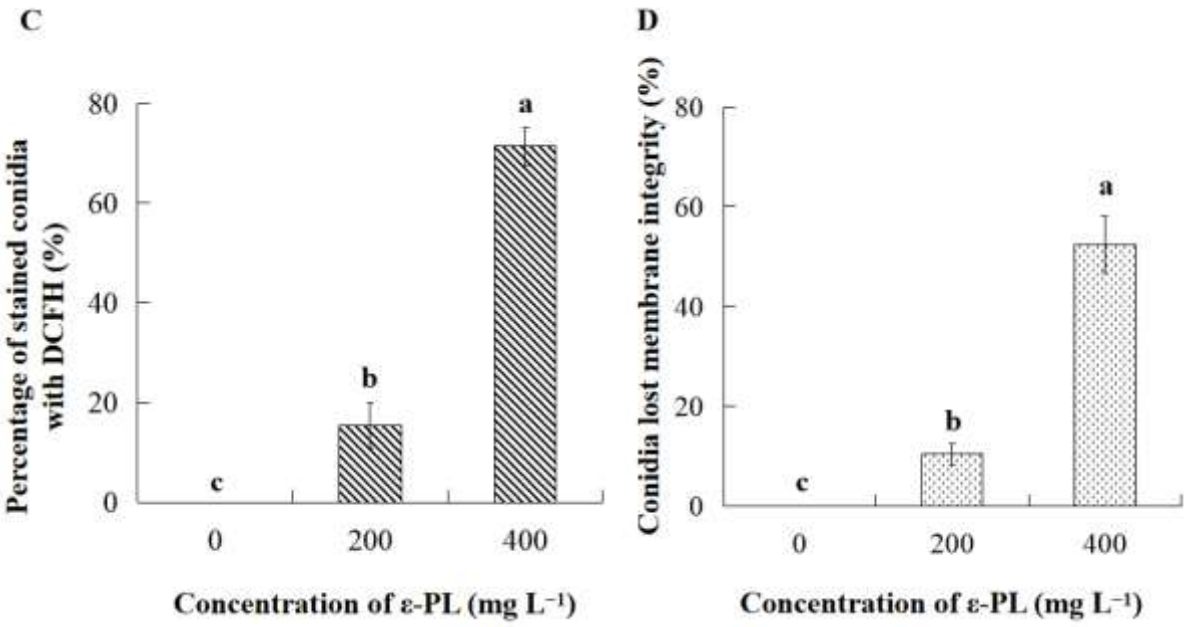
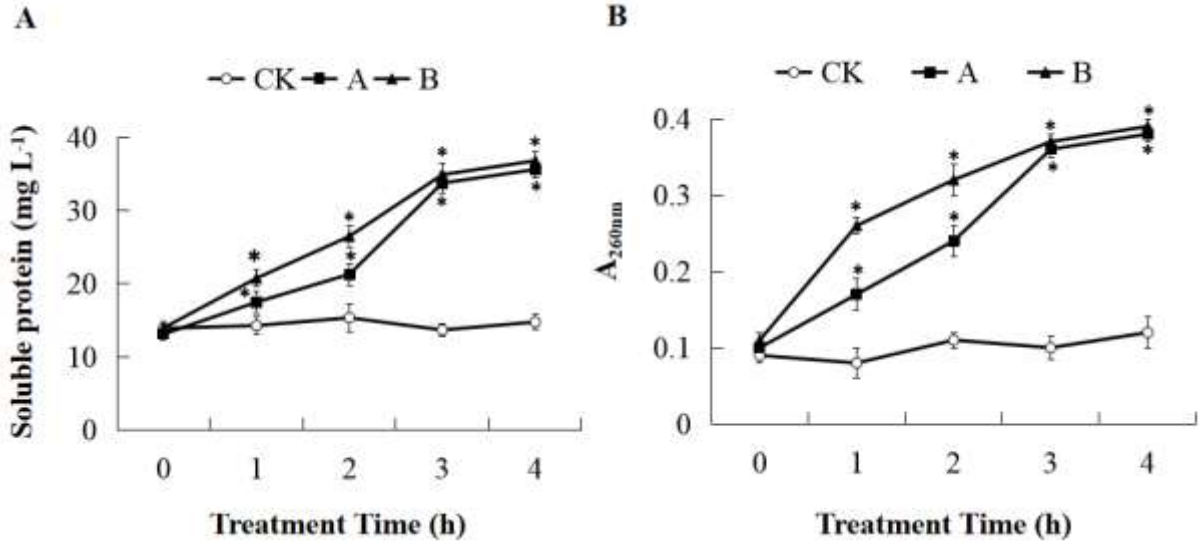


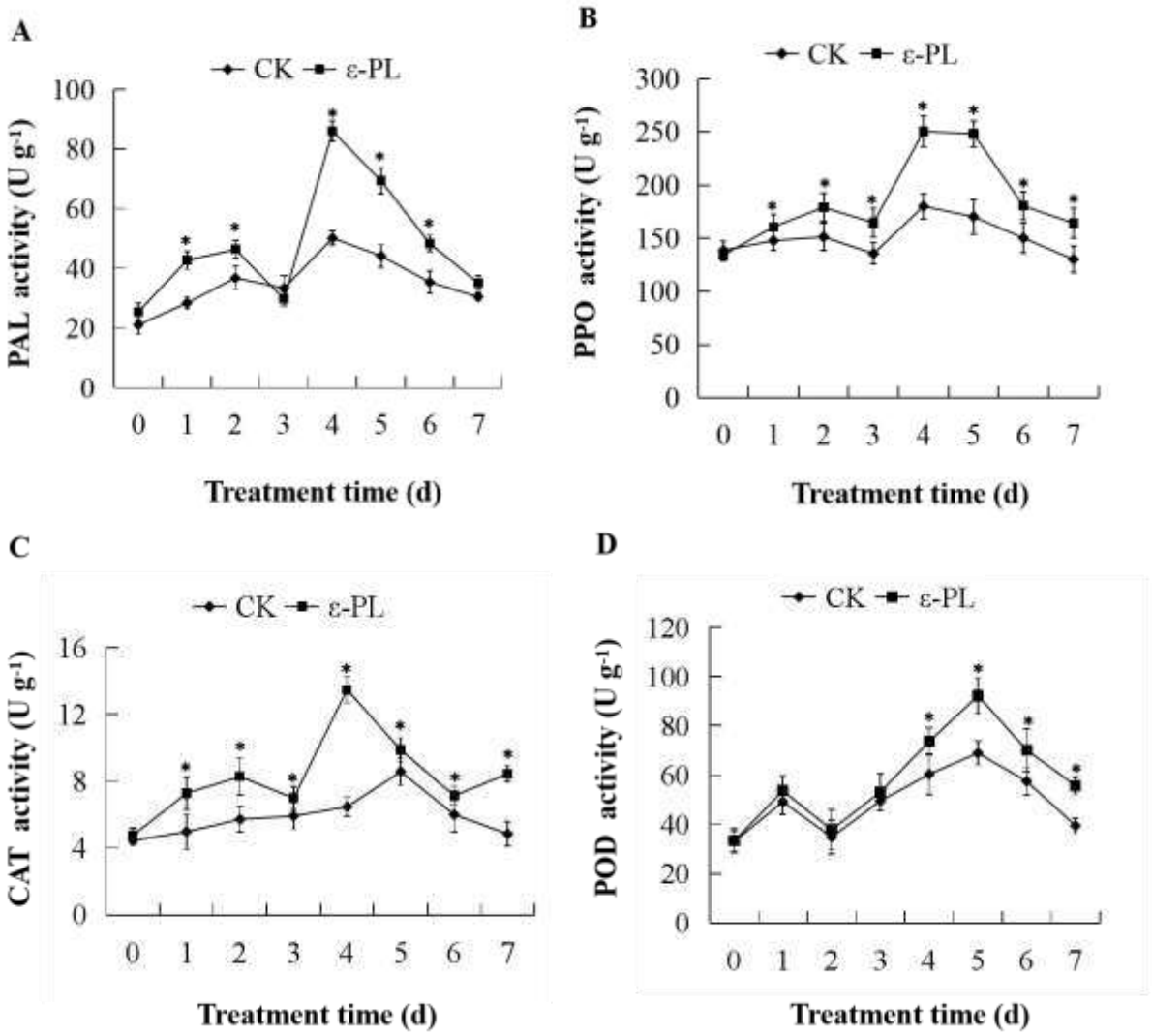
683

684 **Fig.5**



685

686 **Fig.6**



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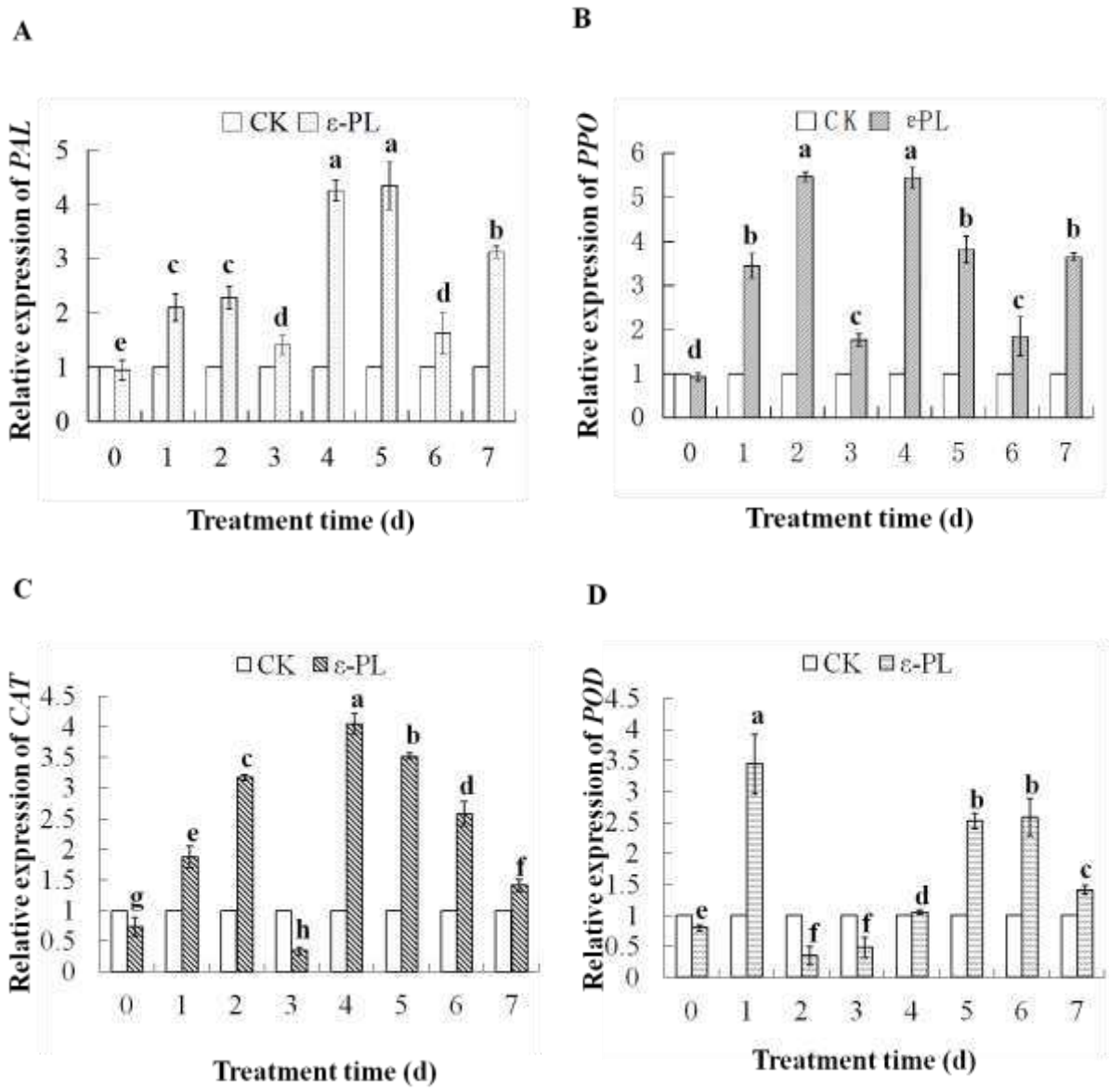
690 **Fig.7**



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