Evaluation and optimization of the protocols for measuring cytochrome P450 activity in aphids

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Abstract

Cytochrome P450 enzymes play major roles in insect detoxification of plant toxins and insecticides. However, measuring P450 activity in aphids has variable success, and a reliable method is not available yet. In this study, we evaluated and optimized the method for measuring P450 activity in aphids using the 7-ethoxycoumarin as the substrate. First, we found that nicotinamide adenine dinucleotide phosphate and protective agents are not needed in the aphid P450 activity assay, and homogenizing the green peach aphid, *Myzus persicae*, in the microplate resulted in significantly higher P450 activities than those in Eppendorf tube. Homogenizing aphids in Eppendorf tube could grind tissues thoroughly and released uncharacterized compounds that could inhibit aphid and pig liver P450 activities, whereas aphids in the microplate likely could not be thoroughly ground and thus released fewer such inhibitors. Then, the microplate homogenization method was optimized to follows: one or two aphids were put into one well of the microplate and ground in phosphate buffer using pipette tips for 20 cycles, followed by addition of 7-ethoxycoumarin, and then incubated for 1 h at room temperature, after which glycine buffer-ethanol mixture was added to stop the reaction. This method is also suitable for the pea aphid, *Acyrthosiphon pisum*, and the bird cherry□oat aphid, *Rhopalosiphum padi*. These results emphasize the importance of considering inhibitory effect of endogenous compounds in insects on their P450 activity and provide one possible method to reduce this inhibitory effect.

Key words: *Acyrthosiphon pisum*, cytochrome P450, detoxification enzymes, 7-ethoxycoumarin, *Myzus persicae*, *Rhopalosiphum padi*

Introduction

Cytochrome P450 enzymes can be found in almost all living organisms. They play important roles in the metabolism of endogenous compounds such as steroids, vitamins, hormones, carbohydrates and fatty acids, and they are also heavily involved in detoxifying exogenous compounds such as drugs, plant toxins, and mutagen (Liu et al., 2015). The P450 gene expression and activity in insects can be induced by plant toxins and further may confer insect resistance to insecticides (Liu et al., 2015). For example, overexpression of P450 gene *CYP6CY3* was essential for the green peach aphid, *Myzus persicae*, in detoxifying nicotine in tobacco as well as neonicotinoid insecticides (Puinean et al., 2010). The elevated P450 activity in insects has been used as a biomarker for certain insecticide resistance (Liu et al., 2015). Therefore, it is important to accurately measure the activities of P450 enzymes in insects.

Some previous studies failed to measure P450 activity in aphids as well as in some other small insects (Philippou et al., 2010; Gottardi et al., 2016), probably because the P450 activity was too low to detect by traditional method. Another possibility is that some uncharacterized compounds, released during homogenization, might inhibit the P450 activity (Orrenius et al., 1971; Wilson & Hodgson, 1972; Gilbert & Wilkinson, 1975; Valles & Yu, 1996). In addition, the P450 activity of mites could only be detected in supernatant after low-speed centrifugation, suggesting that the general P450 activity assays carried out with microsomal fraction prepared from high-speed centrifuged tissue homogenate may not be suitable for all organisms (Pasay et al., 2009). Until now, no universal method for measuring P450 activity in aphids has been established, although in certain aphid species such measurement has been reported (Castaneda et al., 2009).

Most assays measuring P450 activity are based on the transformation of non- or low-fluorescent

64 P450 substrates to highly fluorescent metabolites catalyzed by P450 enzymes (Desousa et al., 1995;
65 Gottardi et al., 2016). Among these substrates, 7-ethoxycoumarin (7-EC, 7-Ethoxy-1-benzopyran-2-one)
66 is the most widely used chemical for measuring P450 dependent 7-ethoxycoumarin-O-dealkylation
67 (ECOD) activity (Gottardi et al., 2016). This substrate has been widely used for measuring P450 activities
68 in insects, fish, mammals, and nematodes, although other substrates may also work (Castaneda et al.,
69 2009; Pasay et al., 2009; Gottardi et al., 2016). Therefore, we chose 7-ethoxycoumarin as the substrate for
70 measuring aphid P450 activity in this study.

71 The ECOD activity in *M. persicae* was difficult to measure because of endogenous inhibitors
72 presented in *M. persicae* homogenate, which could even significantly reduce ECOD activity of rabbit
73 liver homogenate (Philippou et al., 2010). We obtained a much higher ECOD activity by homogenizing *M.*
74 *persicae* in the 96-well microplate than in the Eppendorf (EP) tube. Furthermore, we found that
75 homogenizing *M. persicae* in the EP tube inhibited ECOD activity of pig liver, whereas no inhibitory
76 effect was found when aphids were homogenized in the microplate. Accordingly, we evaluated and
77 optimized the method measuring ECOD activity in *M. persicae* by homogenizing aphids in the microplate.
78 The results suggest that homogenizing aphids in the microplate can significantly reduce the release of
79 inhibitors and is suitable for measuring P450 activity in *M. persicae* as well as some other aphid species.

80

81 **Materials and methods**

82 **Chemicals**

83 Potassium dihydrogen phosphate (KH_2PO_4, CAS: 7778-77-0, purity: ≥ 99.5%), dipotassium hydrogen
84 phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$, CAS: 16788-57-1, purity: ≥ 99.0%), glycerol (CAS: 56-81-5, purity:

≥ 99.0%), and ethanol (CAS: 64-17-5, purity: ≥ 99.0%) were obtained from Guangzhou Jinhuada Chemical Reagent Co., LTD (China). Phenylmethanesulfonyl fluoride (PMSF, CAS: 329-98-6, purity: ≥ 99.0%) was aquired from Beijing Solarbio Life Sciences Co., LTD (China). DL-dithiothreitol (DTT, CAS: 3483-12-3, purity: ≥ 99.0%), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, CAS: 6381-92-6, purity: ≥ 99.0%), and β-nicotinamide adenine dinucleotide phosphate reduced tetra (cyclohexylammonium) salt (NADPH, CAS: 100929-71-3, purity: ≥ 98%) were from Sigma-Aldrich (China). 7-Ethoxycoumarin (7-EC, CAS: 31005-02-4, purity: ≥ 99.0%) were bought from J&K Scientific Ltd. (China) and 7-hydroxycoumarin (7-OH, CAS: 93-35-6, purity: ≥ 98%) were from Shanghai Macklin Biochemical Co., Ltd. (China).

Aphids

Myzus persicae was reared on cabbage plants (*Brassica oleracea* L. var. *capitata*, var. "Qingan 70") under a 14:10 h L/D cycle at 22 ± 2°C and 50% relative humidity. The pea aphid, *Acyrthosiphon pisum*, was reared on broad bean and the bird cherry-oat aphid, *Rhopalosiphum padi*, was reared on wheat under the same conditions.

Comparison of homogenization methods

To measure P450 activity, most studies homogenize insect tissues in a mortar or an EP tube with fitted pestles, which could grind tissues thoroughly. Another method is putting intact insects or their dissected tissues directly into phosphate buffer in a multi-well microplate and then break up with pipette tips or proceed with no further homogenization (Desousa et al., 1995; Gottardi et al., 2016). First, we compared

106 these two homogenization methods, i.e. homogenizing aphids in an EP tube with fitted pestles, and in a
107 96-well microplate with pipette tips. For EP tube homogenization method, adult aphids were placed into a
108 1.5 mL EP tube and homogenized with a plastic pestle in ice cold 100 mM phosphate buffer (pH 7.5)
109 containing protectants (1mM EDTA, 1 mM DTT, 1 mM PMSF and 20% glycerol). The homogenate was
110 used for measuring P450 activity without centrifugation. Then, 2.5 mg equivalent aphid homogenates
111 were added to each microplate well that contained 100 μL 7-EC solution (0.5 mM 7-EC dissolved in 100
112 mM phosphate buffer pH 7.5 containing protectants). For the microplate homogenization method, 2.5 mg
113 adult aphids were placed into each well of the microplate and homogenized in 100 μL 100 mM phosphate
114 buffer (pH 7.5) containing protectants using a 200 μL pipette tip. Then, 100 μL 7-EC solutions (0.5 mM
115 7-EC dissolved in 100 mM phosphate buffer pH 7.5) were added to each microplate.
116 After the mix of aphid homogenates with 7-EC, the reaction was initiated by the addition of 10 μL
117 2.5 mM NADPH into each well. Before the addition of NADPH, the fluorescence intensity of each well (t
118 = 0) was measured with the Tecan Infinite M200 Microplate Reader (Tecan Group Ltd, Mannedorf,
119 Switzerland) using 380 nm excitation and 455 nm emission filters, and was considered as basal
120 fluorescence. The microplates were then incubated at room temperature (22 ± 1^{o}C) and the fluorescence
121 intensity was determined after 1 h. The reaction was stopped by adding 100 μL 50% (v/v) glycine buffer
122 (0.5 M)-ethanol, pH 10.4 and fluorescence (t = 1 h). Relative fluorescence units (RFU) was calculated as
123 RFU = fluorescence (t = 1 h) - fluorescence (t = 0). Three or four replicates were performed for each
124 treatment for all assays in this study.
125
126 **Optimization of the P450 activity assay conditions**

We found that homogenizing *M. persicae* in the microplate resulted in significantly higher RFU; therefore, the following assays were conducted to optimize the microplate homogenization method. First, we tested the necessity of NADPH and protectants (i.e. EDTA, DTT, PMSF, and glycerol) in P450 activity measurement. Three adult *M. persicae* were homogenized in each well of the microplate using pipette tips in 50 μL 100 mM phosphate buffer or with phosphate buffer containing protectants. Then 50 μL 7-EC solutions (1 mM 7-EC dissolved in 100 mM phosphate buffer pH 7.5 with or without protectants) were added to each well. The reaction was initiated by the addition of 10 μL 2.5 mM NADPH or 10 μL phosphate buffer and stopped by adding 100 μL 50% (v/v) glycine buffer (0.5 M)-ethanol after 1 h. The RFU was determined by the microplate reader as described above.

Because we found that NADPH and protectants was not indispensable in measuring aphid P450 activity, these chemicals were not used in the following experiments. To optimize the homogenization method, three adult *M. persicae* were placed in each microplate well and were gently crushed or ground with pipette tips for different cycles. To optimize the number of aphids used in each well, 1, 2, 3, or 5 adult *M. persicae* were homogenized in the microplate and their P450 activities were determined as described above. Time dependence of *M. persicae* P450 activities were determined similarly by using two adult aphids in each microplate well.

Inhibitory effect of *M. persicae* homogenate

Two mg aphid equivalents homogenized in the EP tube without centrifugation were mixed with liver extracts to examine whether the lower P450 activity of aphids homogenized in the EP tube was due to inhibitors released during homogenization. Liver extract was prepared by grinding fresh pig liver tissue in

100 mM phosphate buffer (pH 7.5) using a glass mortar and pestle. The liver homogenate was then centrifuged at 4°C 13,000 g for 15 min and the supernatant was collected and used. To test the heat stability of the endogenous inhibitors, aphid homogenate was heated in boiling water for 5 min and then mixed with liver extract. The RFU was determined and calculated following previous procedure using 7-EC (dissolved in 100 mM phosphate buffer pH 7.5) as substrate.

We also tested the inhibitory effect of the supernatants of aphid homogenate and whether homogenizing aphids in the microplate had such inhibitory effect on liver extract. After being homogenized in the EP tube, aphid homogenate was centrifuged at 4°C 13,000 g for 15 min and the supernatant was collected and used. For microplate homogenization method, aphids were ground in the microplate using pipette tips for 20 cycles and then mixed with liver extract. The RFU was determined and calculated as described above.

Suitability of the microplate homogenization method for *A. pisum* and *R. padi*

To test the suitability and necessity of the microplate homogenization method for measuring P450 activities in *A. pisum* and *R. padi*, ECOD activities in these aphids were determined and compared using different homogenization methods. The inhibitory effects of both aphid homogenates on the P450 activity of liver extract were also examined.

Fluorescence of chemicals involved in the P450 activity assay

One hundred μL 7-EC, 7-OH, or NADPH, dissolved in 100 mM phosphate buffer pH 7.5 with different concentrations, were put into the microplate and the fluorescence was determined with the microplate

169 reader. The phosphate buffer was used as the blank control. The fluorescence of these chemicals was also
170 measured using similar methods after the addition of 100 μL 50% (v/v) glycine buffer (0.5 M)-ethanol,
171 pH 10.4.
172
173 **Statistical analysis**
174 The RFU of *M. persicae*, *A. pisum*, and *R. padi* homogenized with different methods were analyzed using
175 Student's t-test. The influence of NADPH and protectants, grinding degree, number of aphids used in
176 each microplate well, and inhibitory effects of aphid homogenate on ECOD activities were analyzed
177 using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD)
178 tests, at a significance level of $P < 0.05$. All statistical analyses were performed using the IBM SPSS
179 Statistics package 19 (SPSS Inc., Chicago, IL, USA).
180
181 **Results and discussion**
182 **Comparison of homogenization methods**
183 The ECOD activity in *M. persicae* was about three times higher when homogenized aphids in the
184 microplate than in the EP tube (Figure 1; $df = 4$; $t = 8.28$; $P = 0.001$), which agrees with a previous study
185 that failed to detect ECOD activities in *M. persicae* because of endogenous inhibitors released during
186 homogenization (Philippou et al., 2010). In addition, we found that homogenized *M. persicae* in the EP
187 tube (without centrifugation) reduced liver ECOD activities by 90% (Figure 2A; $df = 2, 9$; $F = 427.806$; P
188 < 0.001), and this effect still existed after heating aphid homogenate in boiling water (Figure 2A) or after
189 centrifugation (Figure 2B). According to previous studies, the inhibitory compounds in *M. persicae* could

be eye pigment and/or uncharacterized nucleoprotein (Wilson & Hodgson, 1972; Gilbert & Wilkinson, 1975). However, aphids homogenized in the 96-well microplate showed higher ECOD activities (Figures 1), and did not inhibit liver ECOD activities (Figure 2B). Homogenizing aphids in the microplate may not completely grind aphid tissues and thus likely release little or no endogenous inhibitors. These findings suggest the importance of considering endogenous inhibitors before measuring cytochrome P450 activity in aphids.

Optimization of the ECOD activity assay protocol

Since aphid ECOD activities were not enhanced after the addition of the protectants and NADPH, these chemicals are not indispensable in measuring aphid ECOD activity (Figure 3A; df = 2, 6; F = 3.945; P = 0.081). Aphid tissues homogenized in the microplate possibly generated enough NADPH for the P450 activity assay and thus NADPH was not needed (Desousa et al., 1995; Castaneda et al., 2009). Moreover, the fluorescence of residue NADPH may affect the accuracy of the P450 activity assay and needs further processes to eliminate this influence (Chauret et al., 1999).

Grinding *M. persicae* for 20 cycles in the microplate resulted in relatively higher ECOD activities, whereas higher grinding cycles or crush alone did not lead to higher ECOD activities (Figure 3B; df = 3, 8; F = 2.943; P = 0.099). Homogenizing aphids by crushing alone may limit the contact of aphid P450 enzyme with 7-EC, while more grinding cycles may release more inhibitors, both of which lead to lower ECOD activities. Similarly, three or five aphids in one microplate well did not generate higher ECOD activities than two aphids (Figure 3C; df = 3, 15; F = 5.949; P = 0.007), which may be due to that more aphids in one microplate well lead to higher concentration of inhibitors. Although the RFU of this assay

211 remained linear for 3 h (Figure 3D; R^2 = 0.95), the ECOD activities of different samples could be
212 differentiated in 1 h because of the high sensitivity. In summary, we improved the method measuring
213 aphid P450 enzyme activities as follows: one or two aphids were placed in one well of a 96-well
214 microplate, and ground in phosphate buffer using pipette tips for 20 cycles, followed by addition of 7-EC,
215 and then incubated for 1 h at room temperature before adding glycine buffer-ethanol mixture to stop the
216 reaction.
217
218 **Suitability of the microplate homogenization method for *A. pisum* and *R. padi***
219 The pea aphid, *A. pisum* had only approximately one third ECOD activities when homogenized in
220 the EP tube than in the microplate (Figure 4A; df = 6; t = 3.208; P = 0.018), and this homogenate
221 significantly reduced liver ECOD activities (Figure 4C), suggesting that *A. pisum* released P450 enzyme
222 inhibitors during homogenization in the EP tube. However, the bird cherry-oat aphid, *R. padi*, had no such
223 inhibitory effect on liver ECOD activities (Figure 4C) and showed comparable ECOD activities (Figure
224 4B; df = 6; t = 0.079; P = 0.939) when homogenized with different methods, implying that there may be
225 few or no P450 enzyme inhibitors in *R. padi*.
226
227 **Fluorescence of chemicals involved in the P450 activity assay**
228 The P450 enzyme substrate 7-EC had a low fluorescence intensity (Figure 5A; 1.27 a.u./μM), and its
229 fluorescence intensity increased slightly after the addition of glycine buffer-ethanol mixture (Figure 5A;
230 1.69 a.u./μM). However, 7-OH had a relative higher fluorescence levels (Figure 5B; 12,083 a.u./μM) and
231 the fluorescence intensity increased 3.7 times after the addition of glycine buffer-ethanol mixture (Figure

232 5B; 44,645 a.u./μM). These results suggest that 7-EC is suitable as the P450 enzyme substrate, and the
233 high pH glycine buffer-ethanol buffer can improve the sensitivity of such assay. The NADPH should not
234 be used in this assay, because NADPH is not needed and had a relative high fluorescence intensity
235 (Figure 5C; 9.72 a.u./μM).
236
237 **Conclusion**
238 Although homogenizing aphids in the 96-well microplate reduced the release of endogenous inhibitors, it
239 is difficult to guarantee that no inhibitors were released. However, the samples containing two aphids had
240 about two times higher ECOD activities than one aphid, suggesting that our method can eliminate most, if
241 not all, inhibitors. Nevertheless, the number of grinding cycles and number of aphids in each microplate
242 well should be optimized before specific P450 activity assay. This study suggests the necessary of testing
243 the inhibitory effects of endogenous compounds before measuring P450 activity, and provides one
244 possible method to reduce this inhibitory effect. We used aphids in the present study, but our method may
245 be also suitable for other organisms in the P450 activity assay.
246
247 **Acknowledgments**
248 Funding of this research was supported by the China Postdoctoral Science Foundation Grant (No.
249 2017M613229). We are grateful for the assistance of all staff and students in the Key Laboratory of
250 Applied Entomology, Northwest A&F University at Yangling, Shaanxi, China.
251
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Figure legends

Figure 1 ECOD activities of *M. persicae* homogenized with different methods. Aphids were homogenized in the EP tube with fitted pestles or ground in 96-well microplate using pipette tips. Data are mean ± SE (n = 3). **P < 0.01 (Student's t-test).

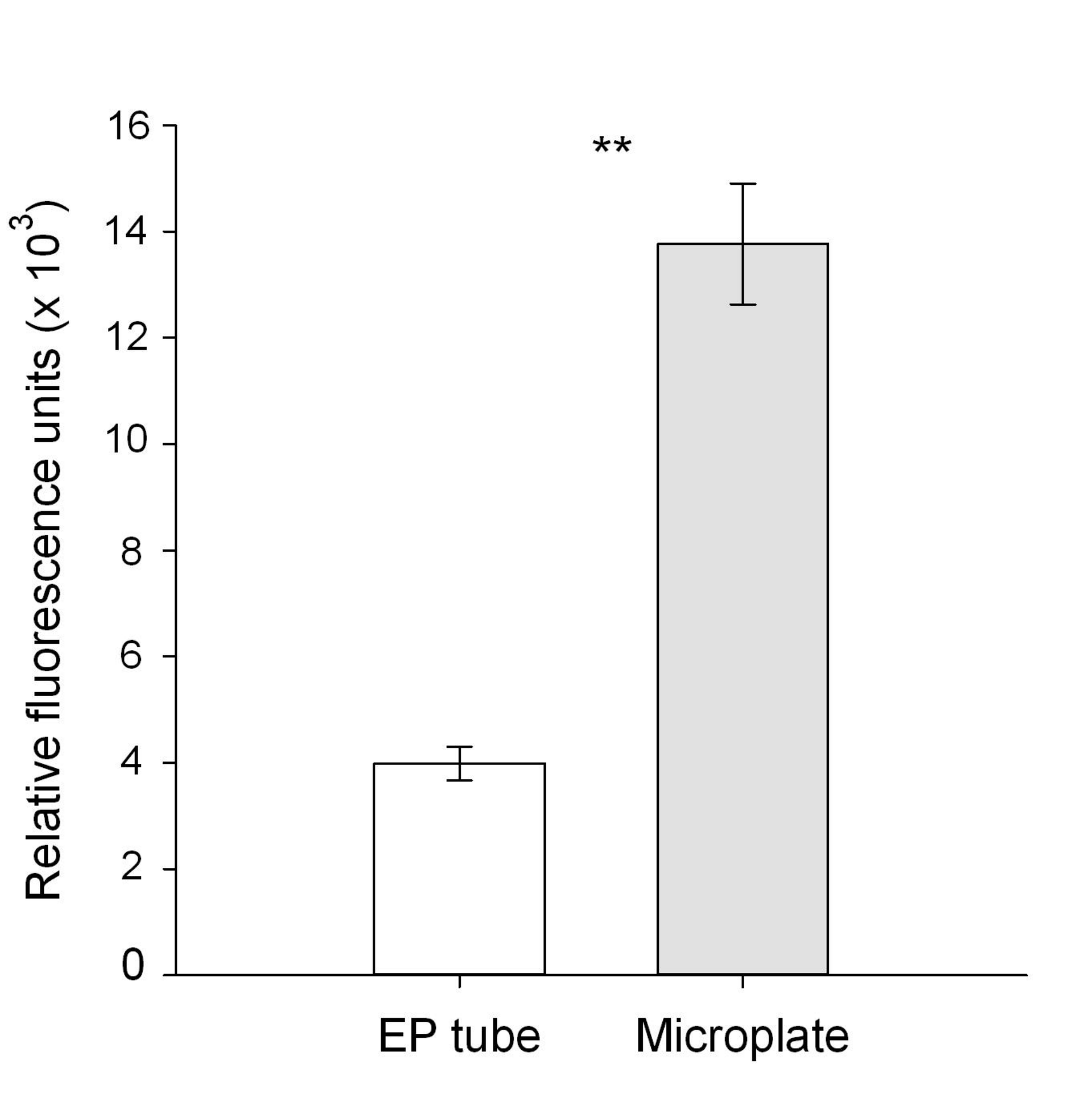
Figure 3 Optimization of the P450 enzyme assay conditions. The influence of (A) NADPH and protectants, (B) grinding degree, (C) number of aphids in each microplate well, and (D) reaction time on ECOD activities of *M. persicae*. Data are mean ± SE (n = 3). Different letters above bars indicate significant difference at P < 0.05 (one-way ANOVA followed by LSD test).

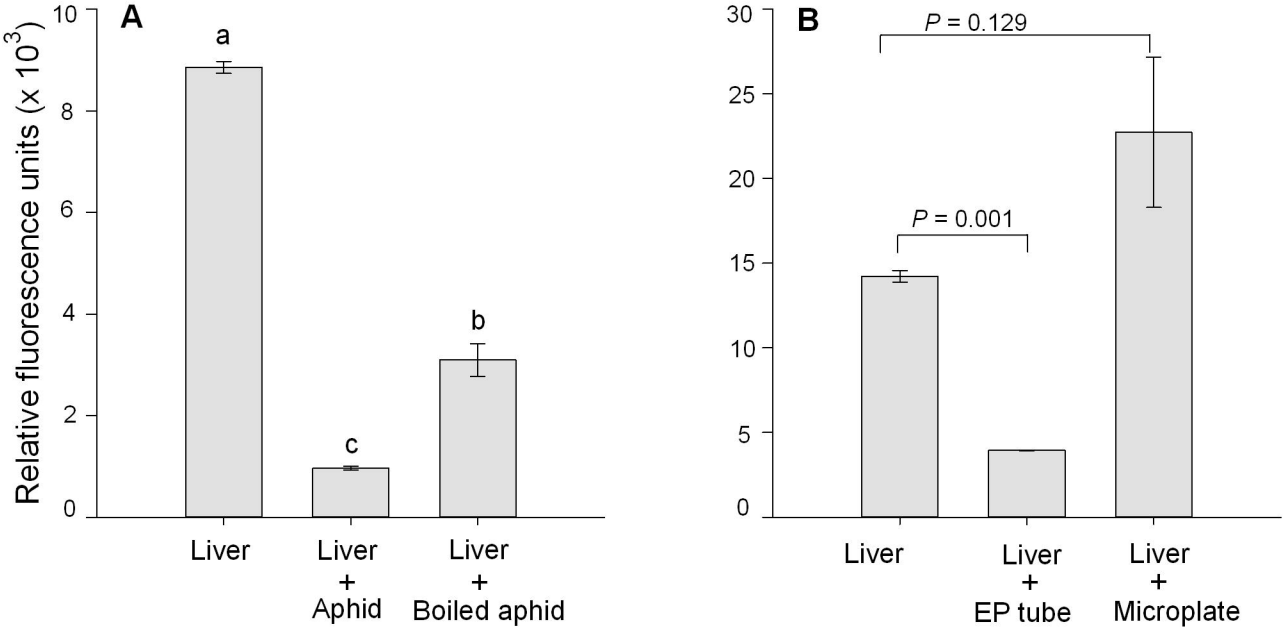
Figure 2 Inhibitory effects of *M. persicae* homogenate on ECOD activity of liver extract. (A) Aphids homogenized in the EP tube (without centrifugation) reduced liver ECOD activity (Liver + Aphid) and the inhibitory effects still existed after heating aphid homogenate in boiling water (Liver + Boiled aphid). (B) Aphids homogenized in the EP tube (after centrifugation) reduced liver ECOD activities (Liver + EP tube), whereas aphids that were directly homogenized in the microplate had no such inhibitory effect (Liver + Microplate). Data are mean ± SE (n = 4). Different letters above bars indicate significant difference at P < 0.05 (one-way ANOVA followed by LSD test). The P values above bars was calculated

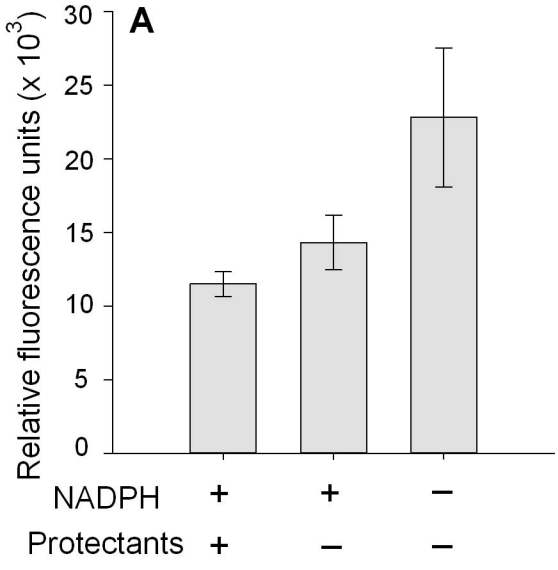
by Student's t-test.

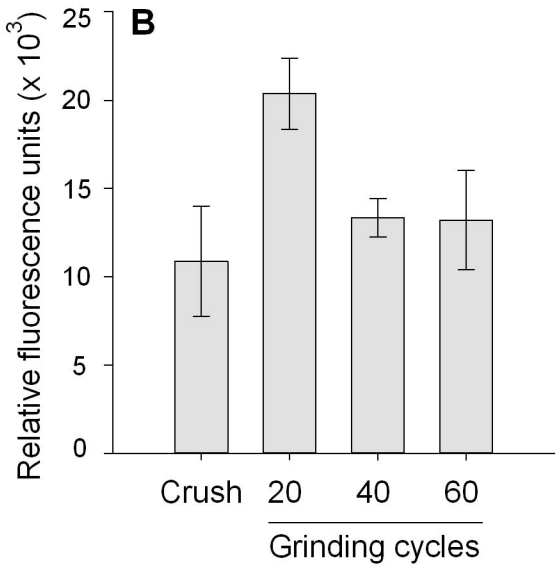
Figure 4 ECOD activities of (A) *A. pisum* and (B) *R. padi* homogenized in the EP tube or microplate and (C) inhibitory effects of aphid homogenate (homogenized in the EP tube) on liver ECOD activity. Data are mean ± SE (n = 4). *P < 0.05 (Student's t-test) and different letters above bars indicate significant difference at P < 0.05 (one-way ANOVA followed by LSD test).

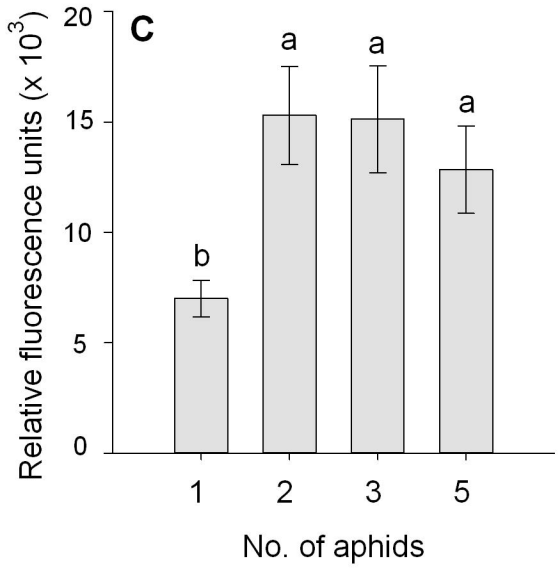
Figure 5 Fluorescence intensity of (A)7-EC, (B) 7-OH, and (C) NADPH before and after the addition of 50% (v/v) glycine buffer-ethanol. Data are mean ± SE (n = 3).

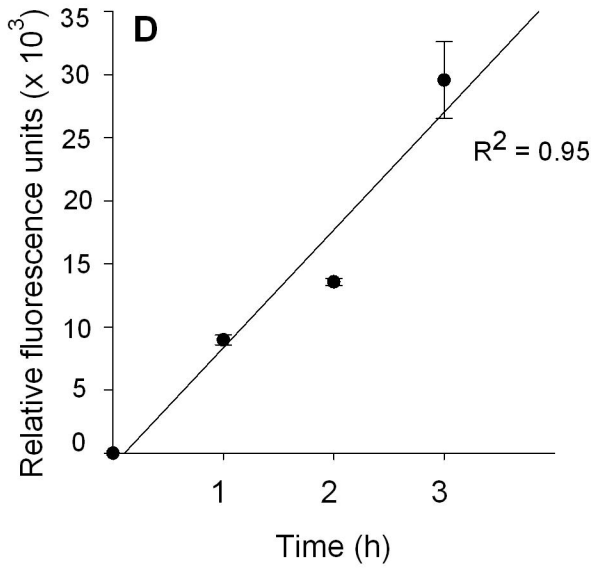

Relative fluorescence units (x 10^3)
16
14
12
10
8
6
4
2
0
**
EP tube
Microplate

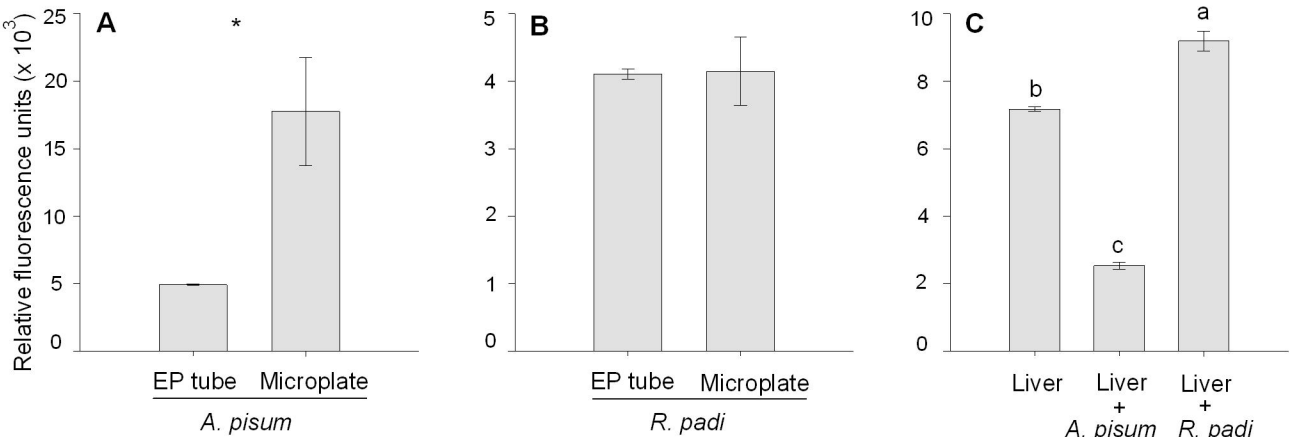

A
Relative fluorescence units (x 10^3)
10
8
6
4
2
0
a
b
c
Liver
Liver + Aphid
Liver + Boiled aphid
B
30
25
20
15
10
5
0
P = 0.129
P = 0.001
Liver
Liver + EP tube
Liver + Microplate

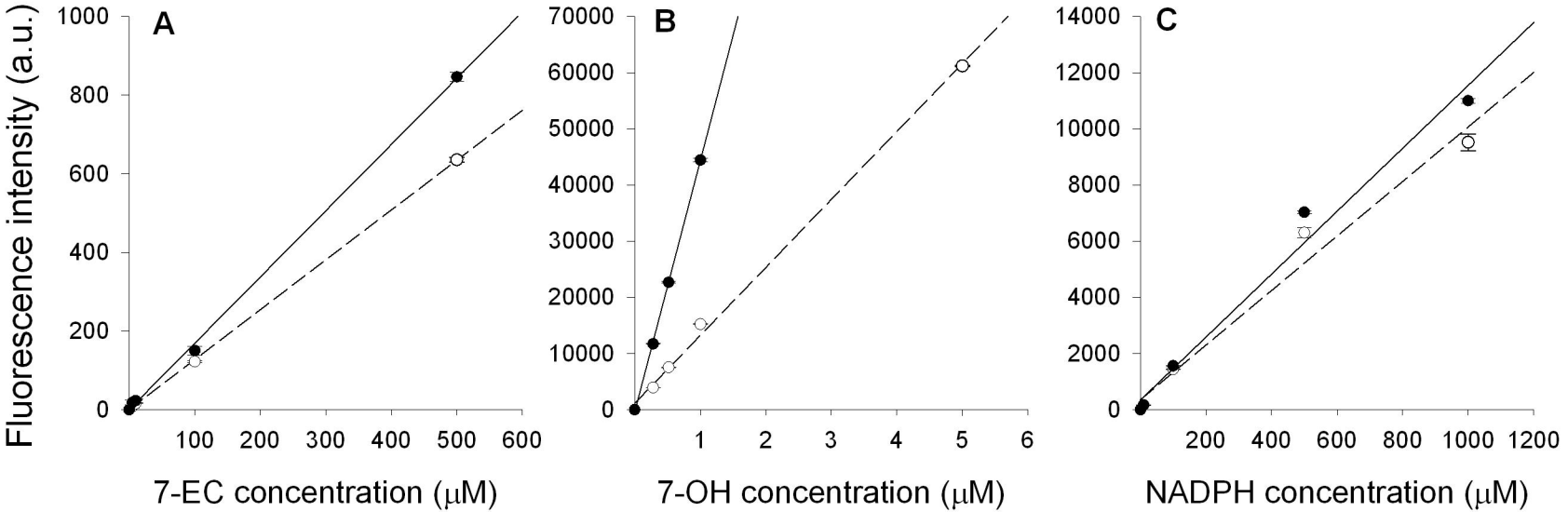

A
Relative fluorescence units (x 10^3)
30
25
20
15
10
5
0
NADPH + + −
Protectants + − −


B
Relative fluorescence units (x 10^3)
25
20
15
10
5
0
Crush
20
40
60
Grinding cycles


C
Relative fluorescence units (x 10^3)
20
15
10
5
0
b
a
a
a
1
2
3
5
No. of aphids


D
Relative fluorescence units (x 10^3)
35
30
25
20
15
10
5
0
R2 = 0.95
1
2
3
Time (h)


A
*
Relative fluorescence units (x 10^3)
25
20
15
10
5
0
EP tube
Microplate
A. pisum
B
5
4
3
2
1
0
EP tube
Microplate
R. padi
C
10
8
6
4
2
0
a
b
c
Liver
Liver + A. pisum
Liver + R. padi


A
B
C
Fluorescence intensity (a.u.)
1000
800
600
400
200
0
100
200
300
400
500
600
7-EC concentration (μM)
70000
60000
50000
40000
30000
20000
10000
0
1
2
3
4
5
6
7-OH concentration (μM)
14000
12000
10000
8000
6000
4000
2000
0
200
400
600
800
1000
1200
NADPH concentration (μM)