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A comparative study of classical and biochemical endpoints for phytotoxicity testing of chlorobenzoic acids

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Abstract: The phytotoxicity of chlorobenzoic acids (CBAs) was studied and the biochemical endpoints' suitability and sensibility was evaluated. Two terrestrial plant species in the same family were exposed to different concentrations of CBAS and tested their germination according to the guideline of Organization for Economic Cooperation and Development (OECP, 1984). The results showed that CBA dose-inhibition rate of classical endpoint had the distinct linear relationship in the range of 10%—50% inhibition rate for root elongation ($p < 0.01$), and the dose variances of CBAs had the greater influence on the inhibition rate of germination than on inhibition rate of root elongation. The CBA dose half effect concentration-inhibition rate of two antioxidant enzyme activity superoxide dismutase (SOD) and catalase (CAT) had the quadratic relationship, and CBA dose-inhibition rate of the peroxides (POD) activity had the linear relationship ($p < 0.05$). Comparing the half effect concentration (EC_{50}) of two kinds of endpoints, the POD activity was more sensitive than classical endpoint, however, SOD and CAT activity were not sensitive in the experiment.

Keywords: inhibition rate; antioxidant enzyme; half effect concentration (EC_{50}); sensibility

Introduction

Chlorobenzoic acids (CBAs) are important chemical products and extensive used as the intermediate and analysis reagent in many industrial fields (Zhang, 2001). Therefore, CBAs may directly and constantly release into environment, and it was also introduced through application of herbicides (Hartmann, 1979) or accumulated with intermediary metabolites as partial degradation products of polychlorinated biphenyls by soil microorganisms (Parsons, 1988). Because of high dissolution in water, CBAs can be easily transfered in soil and groundwater.

Higher plants are essential parts to healthy and balanced ecosystem; the use of higher plants to assess environmental risks has recently gained the attention of scientists in recent decades (Gong, 2001). A number of organizations have set up and developed the consensus guidelines for phytotoxicity tests (OECD, 1984; USEPA, 1989; ISO, 1993). Germination rate and root elongation) as a rapid method for testing phytotoxicity, possess several advantage, such as relative sensitivity, simplicity, low cost suitability for unstable chemicals or samples (Wang, 2001). However, one kind of endpoint is sometimes disturbed by other factors, therefore battery endpoints should be helpful to develop a comprehensive endpoints of toxicity profile for pollutants.

The enzyme is an indispensable factor during vegetation germination. As the main protection mechanism of organism, antioxidant enzyme system is closely related to resisting the adverse circumstances, thus they can reflect the physiological effect of adverse circumstances. There were many reports on qualitative relationship of pollutant dose-antioxidant enzyme activity on terrestrial plants (He, 2003; Liu, 2003; Chu, 2004), however, a few of them focused on the quantitative relationship of pollutant dose-antioxidant enzyme activity.

Many experiments have been conducted to determine the degradation of CBAs and their metabolize process (Layton, 1992; Niedan, 1997; Zhuang, 2003). Hitherto there is little information about phytotoxicity of CBAs (Ajithkumar, 1997).

The aims of this study were (1) to assess phytotoxity of CBA; (2) to evaluate quantitatively the suitability of biochemical endpoints (SOD, POD, CAT) on the CBA phytotoxicity; (3) to compare and analyze the degree of sensitivity between classical and biochemical endpoints.

1 Materials and methods

1.1 Materials

2-CBA, 3-CBA and 4-CBA were obtained from Fluka Corp. Germany (Arabic numerals are chlorine position in CBA molecule, 2-*ortho*, 3-*meta*, 4-*para*; purity of the three chemicals > 99%). The grade of other chemicals and reagents used in the cultivation medium was analytical purity.

Chinese cabbage (*Brassica campestris* L. ssp) and Chinese white cabbage (*Brassica rapa* L. Chinensis Group) seeds were supplied by the Seed Corp. of Shenyang Agriculture University (Shenyang, China).

1.2 Methods

1.2.1 Procedure of seed germination

Before experiments, uniform seeds were sterilized with 0.1% mercuric chloride and were rinsed with distilled water, then they were placed in petri plates (10.0 cm) and moistened by 2-CBA, 3-CBA and 4-CBA respectively at different concentrations (neutralized to pH 7.0). In every petri plate 30 seeds were germinated in the dark at 28 ± 2℃. When germination rate of seeds of control group exceeded 65% and length of root exceeded 20 mm, the experiment was completed. Experiments were performed in triplicate. Controls were maintained on filter paper moistened with distilled water. The inhibition percentages of every endpoint were calculated and compared with those of controls

1.2.2 Extraction and assay of enzymes

Extraction: 0.5 g of seedling fresh biomass was ground to slurry with a mortar and pestle in 10 ml of phosphate buffer (0.05 mol/L, pH 7.8) under cooling condition (ice bath). The homogenates were centrifuged at 12000 r/min at 4℃ for 10 min, and the supernatants were kept at 4℃ prior to assay SOD, POD, and CAT.

Superoxide dismutase assay: The activity of superoxide

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dismutase(SOD) was analyzed by method(Shanghai Academy of Plant Physiology, 1999). The assay medium contained 50 mmol/L phosphate buffer(pH 7.8), 13 mmol/L methionine, 75 μmol/L *p*-nitro blue tetrzolium chloride(NBT), 2 μmol/L riboflavin, 0.1 mmol/L EDTA, and test tubes were placed under light incubator. The absorbance was at 560 nm. One unit of enzyme activity was determined as the amount of the enzyme to reach an inhibition of 50% NBT reduction rate.

Peroxides assay: The activity of POD was measured by a method described in the reference(Zhang, 1990). The assay mixture for the peroxides (POD) comprised of 2.1 ml phosphate buffer (0.1 mol/L, pH 6.8), 0.3 ml 1.6% guaiacol, 0.3 ml 0.04 mol/L H_2O_2 and 0.3 ml enzyme extract. The rate of change in absorbance at 470 nm was determined for 1 min.

Catalase assay: The activity of CAT was analyzed according to Zhou(Zhou, 1995). The activity of CAT was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2. The reaction mixture contained 50 mmol/L phosphate buffer (pH 7.0) and 15 mmol/L H_2O_2.

1.3 Statistical analyse

The two kinds of regression models (linear or curve) were analyzed using SPSS 12.0 and compared feasibility to fit the experimental data. The CBA dose-response of every endpoint was plotted by using SPSS 12.0 and determine the significant test (F-statistic) ($p < 0.05$). The half effect concentration (EC_{50}), plus confidence intervals, were calculated through using plotting equation for those endpoints showing a concentration-response by Math CAD 11.0.

2 Results and discussion

2.1 Effect of CBAs on classical endpoint

The relationship between CBA dose and two kinds of classical endpoints CWC and CC (inhibition rate of germination and root elongation) is shown in Table 1. There is a distinct linear relationship between them.

On the basis of slope of linear equation, the sensitivity of classical endpoints for two kinds of plants in family was similar, however, dose-response of germination was obviously larger than that of root elongation, so it showed the inhibition rate of germination was more sensitive than inhibition rate of root elongation.

The different sensitivities of plant species can be attributed to genetic and physiological differences (Fletcher, 1990). The exact mechanism of inhibition of xenobiotics on higher plants is not clear. In the previous studies, hydrophobicity was proved to be one key factor. But CBAs have a high solution in water, the inhibition of classical endpoints was only related to the chemical properties of CBAs.

Table 1 Relationship of dose-response between classical endpoints and CBAs

Pollutant and concentration range, μg/L	Endpoints	Plotting equation	R^2	*F*-statistic value	*P*
2-CBA(100—200)	CWC*	$Y = 0.42X - 9.10$	0.99	150.91	0.0002
	CC*	$Y = 0.47X - 5.56$	0.97	139.69	0.0003
	CWC**	$Y = 0.32X - 12.33$	0.96	103.78	0.0005
	CC**	$Y = 0.16X + 22.70$	0.95	75.97	0.001
3-CBA(10—100)	CWC*	$Y = 0.76X + 8.86$	0.95	73.58	0.001
	CC*	$Y = 0.66X + 10.99$	0.95	74.62	0.001
	CWC**	$Y = 0.41X + 11.4$	0.95	72.84	0.001
	CC**	$Y = 0.36X + 12.4$	0.98	345.09	0.0001
4-CBA(10—100)	CWC*	$Y = 0.69X - 2.66$	0.98	237.65	0.0001
	CC*	$Y = 0.61X + 8.40$	0.95	88.64	0.001
	CWC**	$Y = 0.50X - 5.43$	0.96	122.81	0.0004
	CC**	$Y = 0.50X + 8.68$	0.86	25.12	0.005

Notes: * germination; ** root elongation; y—inhibition rate of endpoints; x—endpoint concentration

2.2 Effect of CBAs on biochemical endpoints

Biochemical markers are very sensitive and have the advantage of a rapid response. Plants under adverse circumstances will be likely to produce large amounts of free oxide radicals which can induce lipid peroxidation directly or indirectly and cause membrane lesion or damage. Antioxidant enzyme can remove these free oxide radicals and peroxides. Pollutant stress is also regarded as adverse condition.

Table 2 shows that the CBA dose-inhibition rate of two antioxidant enzyme activity(SOD and CAT) had the quadratic relationship, and CBA dose-inhibition rate of the POD activity had the linear relationship ($p < 0.05$). The R^2 of three plotting equation are larger than 0.75, indicating that inhibition of antioxidant enzyme activities were caused by the variance of CBA dose, not test error. The plotting equations is valid by significance test($F > F_{0.05}$).

As a endpoint of phytotoxicity, it should have a stability or suitability, observed easily, and good reappearance, so linear relationship is more advantage than quadratic relationship from experimental view.

2.3 Comparing and analyzing classic endpoints and biochemical endpoints

Sensitivity to toxicants is an important criterion to determine the suitability of a test method that can be adopted and issued into chemicals regulations. The lowest no-observed-effect concentrations (LNOEC) values that will afford to protect species and communities(Girling, 2000). But adverse and comprehensive surrounding generally influenced the endpoints of plant in natural condition, thus the LNOEC value were disturbed by the effect. Therefore EC_{50} was regard as the threshold value of sensitivity in this test.

On the basis of the fitting equation and biochemical knowledge, the EC_{50} value was acquired in Table 3. The EC_{50} of SOD and CAT was out of concentration range in the experiment, however the EC_{50} of POD was in the concentration range and had lower value. The results showed that the SOD and CAT activity was not "good endpoints", and the POD activity was suitable biological endpoints and also more sensitive than classical endpoints.

Table 2 Relationship of dose-response between biochemical endpoints and CBAs

Enzyme	Pollutant and concentration range, μg/L	Plants	Equation	R^2	F-statistic value	P
SOD	2-CBA	CWC	$Y = 0.010X^2 - 3.320X + 255.55$	0.89	12.84	0.035
	(100—200)	CC	$Y = 0.008X^2 - 2.581X + 206.46$	0.90	120.37	0.0015
	3-CBA	CWC	$Y = 0.009X^2 - 1.121X + 26.58$	0.91	15.16	0.025
	(10—100)	CC	$Y = 0.005X^2 - 0.470X + 16.60$	0.87	10.28	0.045
	4-CBA	CWC	$Y = 0.008X^2 - 1.024X + 35.69$	0.90	12.24	0.035
	(10—100)	CC	$Y = 0.007X^2 - 0.487X + 13.91$	0.93	18.89	0.02
POD	2-CBA	CWC	$Y = -13.498 + 0.3835X$	0.95	68.88	0.001
	(100—200)	CC	$Y = -13.940 + 0.355X$	0.84	20.27	0.01
	3-CBA	CWC	$Y = 6.935 + 1.092X$	0.86	25.38	0.007
	(10—100)	CC	$Y = -4.588 + 1.167X$	0.92	47.03	0.0025
	4-CBA	CWC	$Y = 19.810 + 0.718X$	0.93	49.88	0.002
	(10—100)	CC	$Y = 20.283 + 0.758X$	0.90	37.47	0.0035
CAT	2-CBA	CWC	$Y = 0.004X^2 - 1.106X + 157.18$	0.88	10.99	0.04
	(100-1000)	CC	$Y = 0.010X^2 - 2.995X + 247.65$	0.98	101.42	0.002
	3-CBA	CWC	$Y = 0.009X^2 - 0.872X + 33.32$	0.88	11.56	0.04
	(10—100)	CC	$Y = 0.008X^2 - 0.565X + 17.23$	0.90	13.49	0.03
	4-CBA	CWC	$Y = 0.008X^2 - 0.881X + 29.05$	0.92	16.44	0.025
	(10—100)	CC	$Y = 0.009X^2 - 0.815X + 31.06$	0.94	22.17	0.015

Notes: Y-inhibition rate of endpoints; X-endpoint concentration

Table 3 Comparison of EC_{50} between classic endpoints and biochemical endpoints

Endpoint	2-CBA-EC_{50}, μg/L		3-CBA-EC_{50}, μg/L		4-CBA-EC_{50}, μg/L	
	CWC	CC	CWC	CC	CWC	CC
Seed germination	142	119	53	60	72	69
Root elongation	194	168	93	105	111	84
SOD	235	261	146	133	141	119
POD	137	102	39	47	42	39
CAT	261	217	108	109	118	115

3 Conclusions

The two kinds of classical endpoints and one kind of biochemical endpoint(POD) can indicate the phytotoxicity of CBAs, and POD activity is more sensitive than classical endpoints. If dose-response of endpoint has linear relationship, slope of linear plotting equation can be regarded as the "assistant endpoints" to assess the phytotoxicity. Because higher slope means greater variance of dose-response, so the endpoints can be discerned easily during the test.

Classical endpoints express dose-response by apparent character of test plant and some biochemical endpoint can indicate dose-response in physiological level. There is a good orientation to combine all level endpoints(including molecule biomarker) that can "discern" the CBA in natural surrounding as the biomarker of CBA.

Abbreviations: CAT—catalase; CBA—chlorobenzoic acids; CC—Chinese cabbage; CWC—Chinese white cabbage; LOEC—lowest observed effect concentration values; NBT 4—nitro blue tetrazolium chloride; NOEC—no observed effect concentration; ROS—reactive oxygen species; POD—Peroxidase; SOD—superoxide dismutase.

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