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Authors

Huo, Jingqian
Barnych, Bogdan
Li, Zhenfeng
et al.

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Hapten Synthesis, Antibody Development, and a Highly Sensitive Indirect Competitive Chemiluminescent Enzyme Immunoassay for Detection of Dicamba

Jingqian Huo[†,‡,1], **Bogdan Barnych**[‡,1], **Zhenfeng Li**[‡], **Debin Wan**[‡], **Dongyang Li**[‡], **Natalia Vasylieva**[‡], **Stevan Z. Knezevic**[§], **O. Adewale Osipitan**[§], **Jon E. Scott**[§], **Jinlin Zhang**[*,†], **Bruce D. Hammock**[*,‡]

[†]College of Plant Protection, Agricultural University of Hebei, Baoding 071001, P. R. China.

[‡]Department of Entomology and Nematology and UCD Comprehensive Cancer Center, University of California, Davis, California 95616

[§]Haskell Agricultural Laboratory, University of Nebraska Lincoln, Concord, NE, 68728.

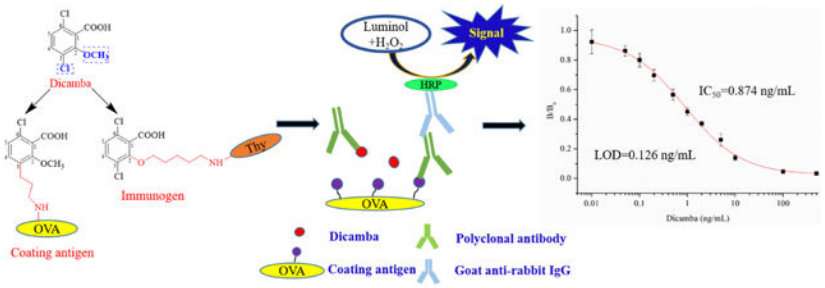
Abstract

Although the dicamba has long been one of the most widely used selective herbicides, now, some US states have banned the sale and use of dicamba because of farmers complaints of a drift and damage to non-resistant crops. To prevent illegal use of dicamba and allow monitoring of non-resistant crops, a rapid and sensitive method for detection of dicamba is critical. In this paper, three novel dicamba haptens with an aldehyde group were synthesized, conjugated to the carrier protein via reductive amination procedure and an indirect competitive chemiluminescent enzyme immunoassay (CLEIA) for dicamba was developed. The assay showed an IC_{50} of 0.874 ng/mL which was over fifteen times lower than the conventional enzyme immunoassay. The immunoassay was used to quantify dicamba concentration in the field samples of soil and soybean obtained from fields sprayed with dicamba. The developed CLEIA showed an excellent correlation with LC-MS analysis in spike and recovery studies, as well as real samples. The recovery of dicamba ranged from 86 to 108 % in plant samples and from 105 to 107 % in soil samples. Thus, this assay is a rapid and simple analytical tool to detect and quantify dicamba levels in environmental samples, and potentially a great tool for on-site crop and field monitoring.

Graphical Abstract

[*]Corresponding authors (Tel: +86-0312-7528575; Fax: +86-0312-7528575; zhangjinlin@hebau.edu.cn) and (Tel: 5307520492; Fax: 5307521537; bdhammock@ucdavis.edu).

[1]These authors contributed equally to this work.



Keywords

dicamba; hapten synthesis; polyclonal antibody; chemiluminescent enzyme immunoassay

Introduction

Dicamba (3,6-dichloro-2-methoxybenzoic acid), a widely used broad-spectrum herbicide first registered in 1967, is mainly applied in corn and triticeae crops for controlling annual, perennial and biennial weeds. [1] Dicamba is also used for the control of weeds in pastures, range land and non-crop areas such as fence rows and roadways when it often is used in combination with a phenoxy herbicide or with other herbicides. [2] Dicamba has been found widespread use because of its high efficiency and low toxicity. The release of dicamba-resistant genetically modified plants (soybean, cotton) by Monsanto is another important factor that promoted an increase worldwide use of dicamba. [3-5] However, dicamba from the old formulations was shown to drift after application. It was reported to vaporize from the treated fields and spread to neighboring non-resistant crops. [6-8] Because of the crop damage and farmers' complaints, Arkansas and Missouri banned the sale and use of dicamba in 2017, [9] and in 2018 EPA implemented additional restrictions on sale and use of dicamba in the USA (https://www.epa.gov/ingredients-used-pesticide-products/registration-dicamba-use-dicamba-tolerant-crops). A lower volatility formulation of dicamba offered by Monsanto was approved by the U.S. environmental protection agency (EPA), but properties of this formulation has not been evaluated by experts outside of Monsanto. In addition, there are reports of suspected illegal use of dicamba. Therefore, it is important to develop an efficient and sensitive analytical method for environmental monitoring which can aid in proper use and monitoring of this herbicide.

At present, the detection and analysis of dicamba (Table 1) are mainly done by chromatographic methods which include gas [10], liquid chromatography [11,12], capillary electrophoresis coupled with ultraviolet (UV) or mass spectrometry (MS) [13-15] detection tandem (UPLC-MS/MS). These methods are not field portable, often require tedious sample preparation, and require expensive equipment. Over years, enzyme-linked immunosorbent assays (ELISA) gained popularity and stand out from the various analytical methods for detection of pesticides and other small molecules.[16-18] The reasons for that are high throughput capacity of ELISA generating quantitative analytical data. Clegg *et al* [19] developed first ELISA for dicamba based on a polyclonal antibody. It has been validated in spiked water samples and its performance was compared to GC-MS. However, the

immunoassay developed by Clegg *et al* had relatively low sensitivity (IC_{50} of 195 ng/mL) which probably due to the fact that dicamba was coupled to the carrier protein *via* carboxylic acid, an important structural feature for recognition of dicamba, and because of homologous nature of their immunoassay. Generally, position and nature of the spacer arm are two important factors that influence immunoassay performance, [20-22] and to which particular consideration is given in the current study. In addition, applying the highly sensitive substrate such as a chemiluminescent substrate is another common method to improve the sensitivity of the immunoassay. The chemiluminescent enzyme immunoassays (CLEIA) are often more sensitive compared to conventional ELISA with colorimetric readout and have been widely used in the analytical fields. [23-26]

The immunoassay with better sensitivity capable to detect dicamba in environmental samples following its application is still needed in order to assess the efficiency of application and evaluation if all field was successfully treated. In addition, the assay with high sensitivity to detect dicamba in the areas where dicamba drift may occur is also of great interest. A previous study on dicamba drift showed that average 0.56 g acid equivalent dicamba/ha (0.1% of the applied rate) was found 21 m away from a treated plot.[27] The same study showed that as low as 0.01 % of dicamba standard application rate noticeably affects development of the non-resistant plants. Taken this together with potential need to dilute samples to reduce matrix effects, an immunoassay with high sensitivity is needed to address the problem of dicamba drift. In this study, we report the design and synthesis of three novel dicamba haptens with the aim to improve the sensitivity of the immunoassay. Two excellent polyclonal antibodies (#1000 and #998) were produced, and a quantitative indirect competitive chemiluminescent enzyme immunoassay (CLEIA) selective to dicamba was developed based on these antibodies. The performance of the CLEIA for dicamba was evaluated on spiked and real soil and soybean plant samples, and validated by LC-MS. The CLEIA developed here provides a sensitive and convenient method for detecting dicamba in environmental samples.

Materials and Methods

Chemicals and Reagents.

The chemicals and reagents used for the synthesis of haptens were of analytical grade and were purchased from Sigma (St. Louis, MO) or Thermo Fisher Scientific (Rockford, IL). Bovine serum albumin (BSA), Ovalbumin (OVA), Thyroglobulin (Thy), 3,3', 5,5'-tetramethylbenzidine (TMB), luminol and 4-iodophenol (PIP) were purchased from Sigma (St. Louis, MO). Goat anti-rabbit IgG-horseradish peroxidase was supplied by Abcam (Cambridge, MA). Standards (dicamba, **1**, and its analogs, 5-hydroxydicamba, **2**; 2,3,5-trichlorobenzoic acid, **3**; 2,3,6-trichlorobenzoic acid, **4**; clopyralid, **5**; picloram, **6**; chloramben, **7** and chlorfenac, **8**) (Figure 1)) were purchased from Sigma (St. Louis, MO), Thermo Fisher Scientific (Rockford, IL) or Chem Service, Inc. (West Chester, PA). OriginPro 8.1 (OriginLab, Northampton, MA) was used for processing of the analytical data.

Synthesis of Haptens

All reactions were carried out under an atmosphere of dry nitrogen. All chemicals purchased from commercial sources were used as received without further purification. Analytical layer thin chromatography (TLC) was performed on Merck TLC silica gel 60 F254 plates. Flash chromatography was performed on silica gel (230–400 mesh) from Macherey Nagel. NMR spectra were recorded on Varian VNMRS 600 or Inova 400 instruments. Multiplicity is described by the abbreviations b=broad, s=singlet, d=doublet, t=triplet, q=quartet, p=pentet, m=multiplet. Chemical shifts are given in ppm. ^{1}H NMR spectra are referenced to the residual solvent peak at $\delta = 7.26$ ($CDCl_3$). ^{13}C NMR spectra are referenced to the solvent peak at $\delta = 77.16$ ($CDCl_3$). HRMS spectra were recorded on Thermo Electron LTQ-Orbitrap XL Hybrid MS in ESI. The synthetic route for haptens is shown in Scheme 1 and synthesis details are listed in the supporting information.

Conjugation of hapten to the protein

Haptens JQ-00–21, JQ-00–24 and JQ-00–25 were coupled to carrier proteins (BSA, OVA or Thy) using Schiff base formation as previously described.[28] Briefly, carrier protein (BSA, OVA or Thy; 50 mg) was dissolved in 10 mL of carbonate buffer (pH = 9). Then, a solution of appropriate hapten (JQ-00–21, JQ-00–24 or JQ-00–25; 0.05 mmol) in DMSO was added with gentle stirring. The mixture was stirred for 1 h at room temperature (RT) followed by addition of 100 μL of 5 M cyanoborohydride in 1 N NaOH. The reaction mixture was allowed to react for 3 h at RT (Scheme 2). The resulting conjugates were dialyzed in PBS over 72 h at 4 °C, and stored at −20 °C for further use. The hapten-Thy conjugate was used for immunization, and hapten-BSA and hapten-OVA were used as coating antigens.

3,6-dichloro-2-methoxybenzoic acid (dicamba, JQ-00–26) was coupled to carrier protein (OVA) using a diimine carbonization method. [19] Briefly, a mixture of 3,6-dichloro-2-methoxybenzoic acid (22.1 mg), DMF (500 μL), *N*-hydroxysuccinimide (NHS) (11.5 mg) and *N*, *N*'-dicyclohexylcarbodiimide (DCC) (20.6 mg) was stirred overnight at RT. The supernatant was collected by centrifugation at 13800 g for 5 min and then added dropwise to the 10 mL solution of OVA (112.5 mg) in phosphate buffer (pH = 8.0). The reaction was continued at RT for 4 h, and then the resulting conjugates were dialyzed in PBS over 72 h at 4 °C, and stored at −20 °C for further use.

Production of the Antibody against Dicamba

The immunogens JQ-00–21-Thy, JQ-00–24-Thy, JQ-00–25-Thy were used to produce the polyclonal antibodies #997, #998, #999, # 1000, # 1001 and #1002, respectively. Antagene Inc (Santa Clara, CA) services were used for the rabbit immunizations according to their protocol. Briefly, two New Zealand white rabbits were immunized with each of the immunogens emulsified with complete Freund's adjuvant. The animals were boosted with an additional 100 μg of immunogen that was emulsified with Freund's incomplete adjuvant. Booster injections were given at 20 days intervals. The rabbits were bled 10 days after the forth immunization, and the serum was collected.

Indirect Competitive inhibition ELISA and CLEIA.

A checkerboard procedure was first used to determine the optimal dilution of coating antigen and antibody.

ELISA.—A microtiter plate was coated with 100 μL/well of coating antigen in carbonate buffer (pH 9.6) overnight at 4 °C, and then was blocked with 3% skim milk in PBS (10 mM, pH 7.4). The plate was washed three times with PBST (PBS containing 0.05% Tween 20), and then 50 μL of dicamba standard (or sample) and an equal volume of the antibody solution all dissolved in PBS were added to the wells and incubated for 1 h at RT. The plate was washed five times with PBST and 100 μL/well of goat anti-rabbit IgG-horseradish peroxidase was added at 10000-fold dilution before incubation for 1 h at RT. After washing five times with PBST, 100 μL/well of the TMB substrate solution (12.5 mL of 100 mM, pH 5.5 citrate-acetate buffer containing 200 μL 0.6% TMB dissolved in DMSO and 50 μL 1.0% H_2O_2) was added and the plate was incubated for 15 min at RT. Finally, the reaction was stopped by adding 50 μL/well of 2 M H_2SO_4, and the absorbance was read at 450 nm on an Infinite M1000 PRO.

CLEIA.—The procedure of the CLEIA was similar to that of ELISA. The microtiter plate used in CLEIA was 96 well white microplate and the blocking agent was 2% BSA. After the competitive reaction and washing five times with PBST, 100 μL/well of the luminol substrate system (1 mL of 125 mmol/L luminol in DMSO, 1 mL of 125 mmol/L PIP in DMSO, and 10 μL of 30% H_2O_2 mL added to 8 mL of 0.05 mmol/L Tris-HCl buffer, pH 8.5) was added and the chemiluminescence intensity (relative light units, RLU) was determined using an Infinite M1000 PRO.

Cross-Reactivity (CR)

The selectivity of the antibodies #1000 and #998 was evaluated by testing the cross-reactivity (CR) of a set of dicamba structural analogs. The relative CR was calculated by the following formula:

$$\mathrm{CR}\,(\%) = [\mathrm{IC}_{50}(\text{dicamba}) \,/\, \mathrm{IC}_{50}(\text{tested compound})] \times 100\,.$$

Analysis of Spiked Samples

The spike and recovery study were performed using soybean plants and soil. These blank samples were not sprayed with dicamba and proved to be free of dicamba by LC-MS.

Soybean leaves were frozen with liquid nitrogen, grinded and fortified with dicamba (1 mg/mL in methanol) to final concentrations of 20, 50, 150 ng/g. Soil samples were fortified with dicamba (1 mg/mL in methanol) to final concentrations of 5, 15, 45 ng/g and mixed well. These fortified samples (1.0 g) were extracted using 2 mL of 20 mM PBS containing 50% methanol. After vortexing, the mixture was centrifuged at 1500 g for 15 min, the supernatants were collected and diluted with 20 mM PBS. All the spiked samples were passed through a 0.22 μm filter and then subjected to the CLEIA and LC-MS.

For the LC-MS procedure, samples were injected to an Agilent SL liquid chromatography system and the separation was carried out on a Kinetex C18 column (30 mm×4.6 mm, 2.6 μm). The column temperature was set up at 50℃. Water (solution A) and acetonitrile containing 0.1% (v:v) acetic acid (solution B) were used as the mobile phase with a flow rate of 0.6 mL/min. The volume of sample injection was 5 μL, and the running time was 3 min. The gradient is given in Table S-1.

The LC system was connected to a 4000 Qtrap mass spectrometer. The instrument was operated in a negative ESI mode and multiple reaction monitoring mode. The optimized ion source parameters and MRM method are shown in Table S-2 and S-3, respectively. 12-(3-cyclohexyl-ureido)-dodecanoic acid with final concentration at 200 nmol/L was mixed with analytes and an internal standard to account for ionization suppression.

Analysis and Validation of real samples based on CLEIA and LC-MS

The dicamba resistant soybean plant was sprayed with 56 g/ha, 5.6 g/ha and 0.56 g/ha of dicamba, and the soil samples collected from the same dicamba treated soybean field. The amounts of dicamba in the soybean and soil samples were analyzed by the CLEIA and LC-MS at the same time. The extraction and analysis followed the same procedures with the spiked samples.

Results and Discussion

Design and Synthesis of Haptens

Dicamba is a small molecule therefore it must be conjugated to a large carrier protein in order to elicit an immune response. Generally, it is important to preserve the key functional groups of the target compound for generating a specific antibody, and therefore it is prudent to attach the handle as distal as possible from the key functional groups. [29] Usually, the length of the linker between the hapten and carrier protein is 3–5 carbon atoms. The dicamba molecule contains a carboxylic group which can be directly conjugated to the carrier protein to produce antibodies. Although this method is simple and requires no synthetic chemistry, such a strategy may result in antibodies with low sensitivity due to significant structural differences between free and conjugated dicamba. Coupling the dicamba *via* carboxylic group may also lead to antibodies that mainly recognize the chlorobenzene part of the antigen while the carboxylic acid functionality will be poorly recognized by antibody. A polyclonal antibody developed using the immunogen generated by above mentioned conjugation method was previously developed and its IC_{50} for dicamba was about 200 ng/mL. [19] We hypothesized that exposing dicamba's carboxylic group in antigens may result in antibodies with better characteristics allowing for development of more sensitive immunoassay for this pesticide. In addition, retaining a polar carboxylic acid will reduce the chance that the hapten will fold back into the hydrophobic protein core.

Reductive amination, a common method for the conjugation of haptens to carrier proteins, can be easily performed by reacting the aldehyde group of hapten with the amino group of protein followed by reduction of the labile Schiff base intermediate into a stable secondary amine in the presence of sodium cyanoborohydride.

In this research, three novel haptens of dicamba were designed and synthesized. All haptens had an aldehyde group that was used for the conjugation to the carrier protein by the above-mentioned method. The conjugation of the three haptens with BSA (Figure S1) showed that about 5 to 6 haptens were conjugated per molecule of protein. As a result of this conjugation, the carboxylic group, a key group in the structure of dicamba was exposed following conjugation to the surface of the protein. We also synthesized the previously described coating antigen *via* direct conjugation of dicamba to OVA.

Screening of the Sera and Coating Antigen Combinations

The titers of the six antisera against seven coating antigens were measured. As shown in Table 2, all antisera had low titers to coating antigen JQ-00–26-OVA, which indicated that the carboxylic group in the coating antigen was important for the recognition of the antiserum. Antiserum #1001 and #1002 generated from immunogen JQ-00–25-Thy had no or low titer to all the heterologous coating antigens, but the antiserum #999 and #1000 generated from immunogen JQ-00–24-Thy had high to moderate titers to the heterologous coating antigens. The difference between JQ-00–25-Thy and JQ-00–24-Thy is in the length of the linker, pointing out on the effect of this factor on the antibody specificity. The antisera #997 and #998 generated from JQ-00–21-Thy also had high to moderate titers to the homologous and heterologous coating antigens even when the antiserums were diluted to 10000-fold.

The combinations of antiserum and coating antigen that had good recognition with each other were screened in a three-point competitive format (0, 50 and 500 ng/mL dicamba). The results show (Figure 2) that some combinations had good inhibition with dicamba and the pairs showing inhibition of 50% or over with 50 ng/mL of dicamba were then tested in an 8 points competitive format (Table 3). From the resulting data, we could see that the IC_{50} value for homologous pair was generally higher than that for heterologous assays. For example, in the homologous competitive assay of serum #1000, the IC_{50} value was 220.8 ng/mL (JQ-00–24-BSA), whereas the IC_{50} was 12.3 ng/mL in the heterologous assay (JQ-00–21-OVA). The combinations of #1000/JQ-00–21-OVA and #998/JQ-00–24-OVA were chosen for the following studies because they showed the highest sensitivity.

After optimization of antiserum and coating antigen concentrations the following IC_{50}s were obtained for the above two combinations: 26.9 ng/mL (#998/JQ-00–24-OVA; Figure 3) with the linear range of 3.85–188.17 ng/mL and 14.7 ng/mL (#1000/JQ-00–21-OVA; Figure 4) with the linear range of 3.44–62.9 ng/mL.

The Indirect Competitive Chemiluminescent Enzyme Immunoassay for Dicamba

Many reports have shown that the sensitivity of immunoassays could be significantly improved using the chemiluminescent readout. Therefore, in order to improve the sensitivity of our assay, a competitive chemiluminescent enzyme immunoassay (CLEIA) was developed based on the combination of antiserum #1000 and coating antigen JQ-00–21-OVA which had the highest sensitivity according to the results of ELISA. It is known that the assay parameters such as pH, ionic strength, organic solvent and others influence immunoreactions. Therefore, these parameters were optimized with the goal to decrease the

IC_{50} and increase the ratio of the maximum relative chemiluminiscence (RLU_{max}) to IC_{50} (RLU_{max}/IC_{50}). The best combination fold of antiserum concentration (diluted 6000-flod) and coating antigen (diluted 20000-flod, 175 ng/mL) was determined first using the checkerboard titration. The CLEIA for dicamba was carried out with four different concentrations of PBS, and the results (Figure S2) showed that the chemiluminescence intensity and the sensitivity of the assay are influenced by ionic strength, and the lowest IC_{50} and the highest RLU_{max}/IC_{50} were obtained at 20 mM PBS. Next, the effect of pH on the performance of CLEIA was determined (Figure S3) and the higher IC_{50}s were observed at pH 5 and 6 (IC_{50}=3.91 and 3.95 ng/mL, respectively). Overall the assay showed the best performance at pH 7.4. Because of the relatively weak effect on the immunoreaction, methanol is often used in ELISA to improve the solubility of analyte. In order to evaluate the effect of methanol on the performance of CLEIA, four different PBS solutions containing methanol were studied. As shown in Figure S4, negligible effects on CLEIA were observed at the methanol concentration of 10%. To summarize, the optimal parameters for the CLEIA performance were 10% methanol, pH 7.4, and 20 mM PBS.

A standard curve was established using the optimal conditions obtained from the above study for CLEIA (Figure 5). The standard curve had a good correlation coefficient of 0.997 and a limit of detection of 0.126 ng/mL. This assay had an IC_{50} of 0.874 ng/mL with a linear range of 0.131–5.818 ng/mL. The IC_{50} of CLEIA was over fifteen times lower than that of ELISA (IC_{50}=14.7 ng/mL).

Cross-Reactivity (CR)

Although antibodies #1000 and #998 were obtained using different immunogens, they had similar CR. As shown in Table 4, the antibodies were rather specific toward dicamba, since negligible CR were observed to all compounds except for structurally close 2,3,6-trichlorobenzoic acid (52% for antibody #1000 and 33% for antibody #998). In the previous [19] reported study, the antibody cross-reacted with 5-hydroxydicamba, 2,3,5-trichlorobenzoic acid and 2,3,6-trichlorobenzoic acid about 9.3%, 8.4% and 12.8%, respectively. Although, the compound 2,3,6-trichlorobenzoic acid had a high CR, currently it is not widely used for the weed control and is not likely to be found in environmental samples.

Matrix Effect

Matrix effects are important factors to be considered in immunoassay. Matrix often has a significant effect on the performance of the immunoassay, which then alter the quality of the results. [30] Dilution of the sample with assay buffer is the most common method to reduce or eliminate the matrix effects on immunoassay. Here, the dicamba-resistant soybean and soil were selected for matrix effect evaluation. The blank samples were confirmed to be free of dicamba by LC-MS (LOQ=0.1 ng/mL, LOD=0.03 ng/mL) analysis. Soybean leaves and soil samples were diluted 10-, 20-, 40-, 80-, 160-fold in the assay buffer, respectively. Serial dilutions of dicamba standards were prepared in above mentioned diluted samples. The results (Figure 6A) showed that the RLU_{max} of 10-fold and 20-fold diluted soybean samples were lower than that of other dilutions. Additionally, the 10-fold diluted soybean sample had higher IC_{50}, which indicated that higher concentration of the soybean matrix affected antibody binding. The maximum chemiluminescence intensity and IC_{50} values had no

significant difference among the 40-, 80-, 160-fold diluted matrix samples and assay buffer. Thus, a 40-fold dilution factor was chosen for the developed assay. The soil matrix results (Figure 6B) showed that neither maximum chemiluminescence intensity nor IC_{50} values were significantly affected, indicating that the assay method developed in this study was resistant to soil matrix effects.

Validation Study

The accuracy and reliability of the developed CLEIA for detecting dicamba were evaluated by applying this method for quantification of dicamba in spiked soybean plant and soil samples that were confirmed to be free of dicamba by LC-MS. The soybean and soil were spiked with three different concentrations of dicamba and analyzed by both CLEIA and LC-MS. As shown in Table 5, the average recovery rate from the soybean plant measured using the CLEIA and LC-MS ranged from 86 to 108% and 76 to 117%, respectively. For the soil, the average recovery rate ranged from 105 to 107% (CLEIA) and 107 to 116% (LC-MS). It is worth noting that developed CLEIA method provides quantitative data on total amount of dicamba present in soil, which may differ significantly from the amount bioavailable.

In addition, eight soybean plant samples and six soil samples collected from the field sprayed with dicamba were analyzed with CLEIA and LC-MS. Soybean samples 1–3 were sprayed with 1/10, 1/100, 1/1000 of the dicamba standard rate (560 g/ha) and were collected 1 d after treatment. Soybean samples 4–8 were sprayed with 1/10 of the dicamba standard rate and were collected 7 d, 14 d, 21 d, 39 d and 67 d after treatment, respectively. Soil samples 1–3 were sprayed with 1/10, 1/100, 1/1000 of the dicamba standard rate and were collected 1 d after treatment. Soil samples 4–6 were sprayed with 1/10 of the dicamba standard rate and were collected 7 d, 14 d and 21 d after treatment, respectively. As shown in Table 6, correlation was observed between these two methods. These data show that concentration of dicamba decreases over time after application, consistent with previously reported half-life time of 1 to 4 weeks. [31] Alternatively, the observed time-dependent decrease of dicamba concentration might be at least partially due to its drift, however this factor was not evaluated in the current study. Most importantly, the developed CLEIA method was able to detect dicamba on soybean treated at 1/1000 dicamba standard application rate (560 g/ha), the average drift concentration found 21 m away from the treated field and which can cause slight abnormalities in non-resistant plants.[27]

A good correlation between the CLEIA and LC-MS results were observed for both spike and recovery studies and real samples. Thus, the developed CLEIA method showed good accuracy and reliability for the detection and quantification of dicamba in environmental samples. This method will be instrumental in evaluating drift propensity of new dicamba formulations as well as for rapid analysis of large number of environmental samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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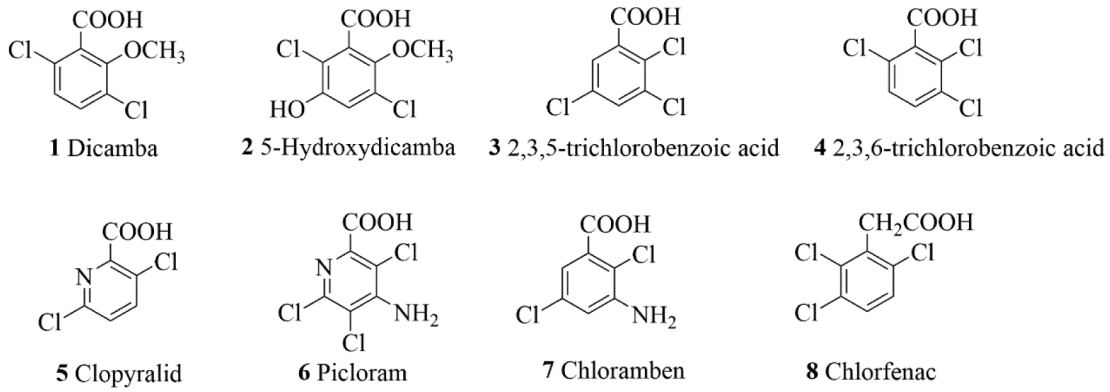


Figure 1.

Structures of dicamba **1**, and structurally related compounds **2-8** tested for cross-reactivity.

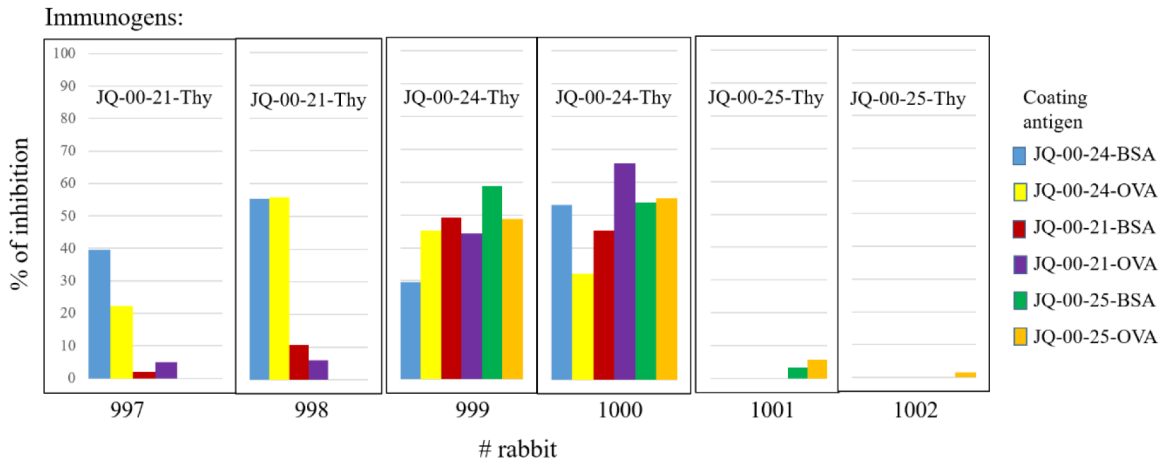


Figure 2.

Screening for successful pairs of coating antigen/serum. Criteria of success is 50% of inhibition and over, at 50 ng/mL dicamba. Absence of the bar indicates that selected serum did not recognize corresponding coating antigen.

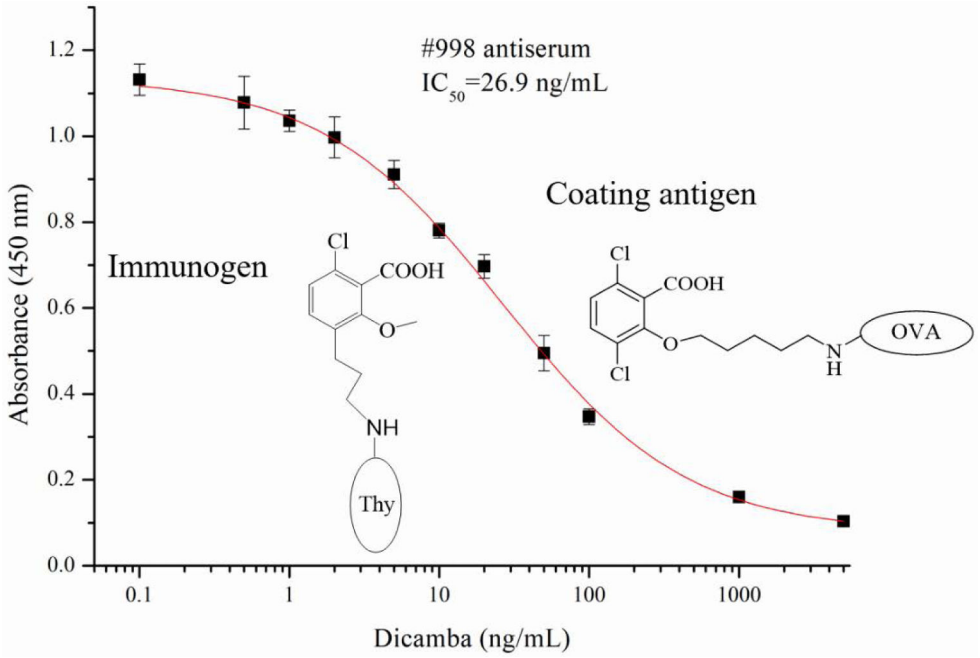


Figure 3.

Inhibition curve for dicamba using antiserum #998. Coating antigen JQ-00-24-OVA 0.35 μg/mL, antiserum 1:4000; Goat anti-rabbit IgG-horseradish peroxidase 1:10000. Each point was tested in triplicate.

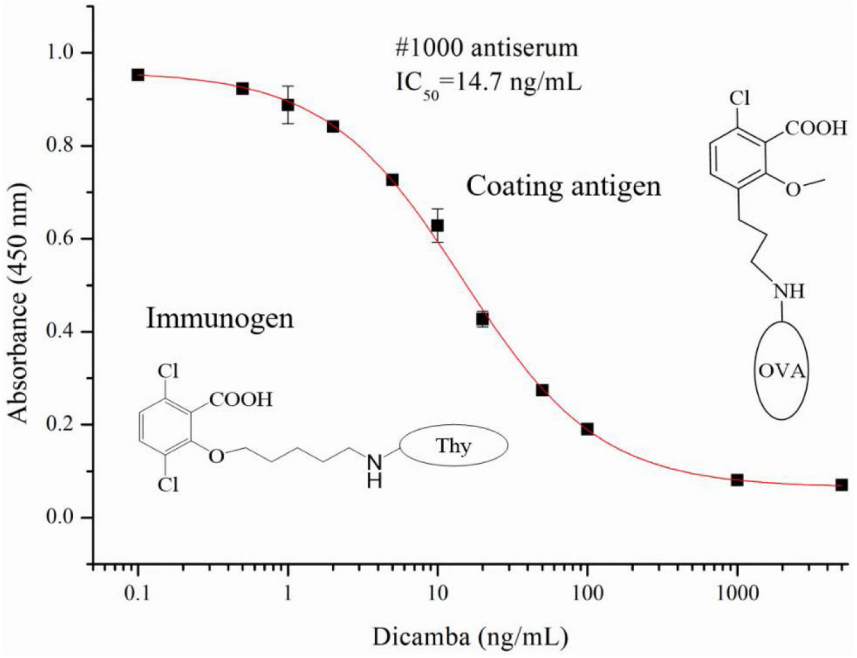


Figure 4.

Inhibition curve for dicamba using antiserum #1000. Coating antigen JQ-00-21-OVA 0.5 μg/mL, antiserum 1:2000. Goat anti-rabbit IgG-horseradish peroxidase 1:10000. Each point was tested in triplicate.

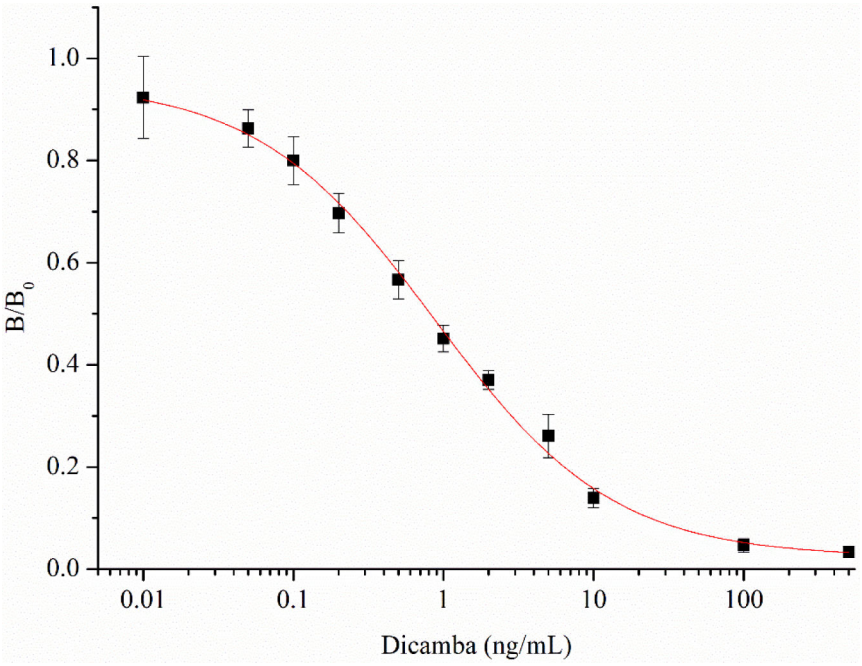


Figure 5.
Standard competitive binding curve of antiserum #1000 based CLEIA for dicamba under optimized parameters. Coating antigen JQ-00-21-OVA, 175 ng/mL. antiserum #1000, 1:6000.

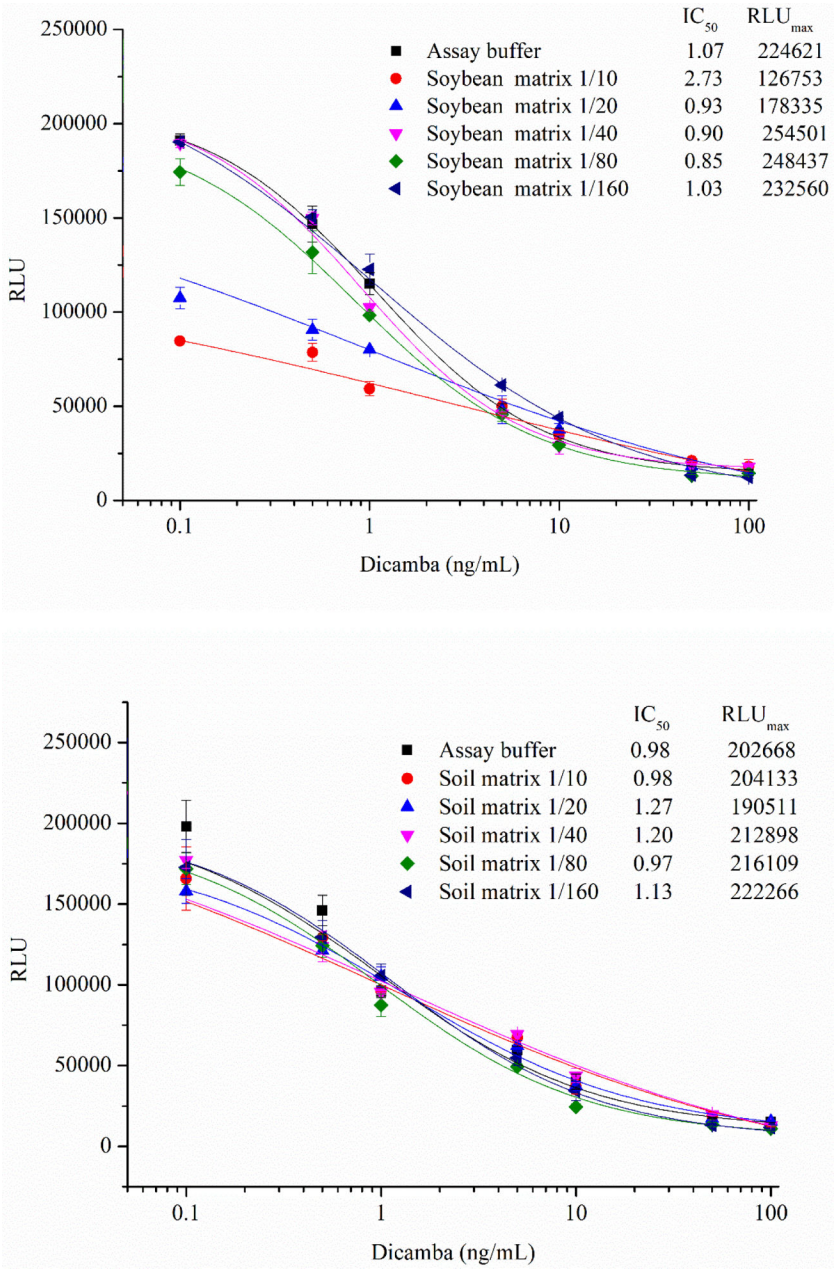
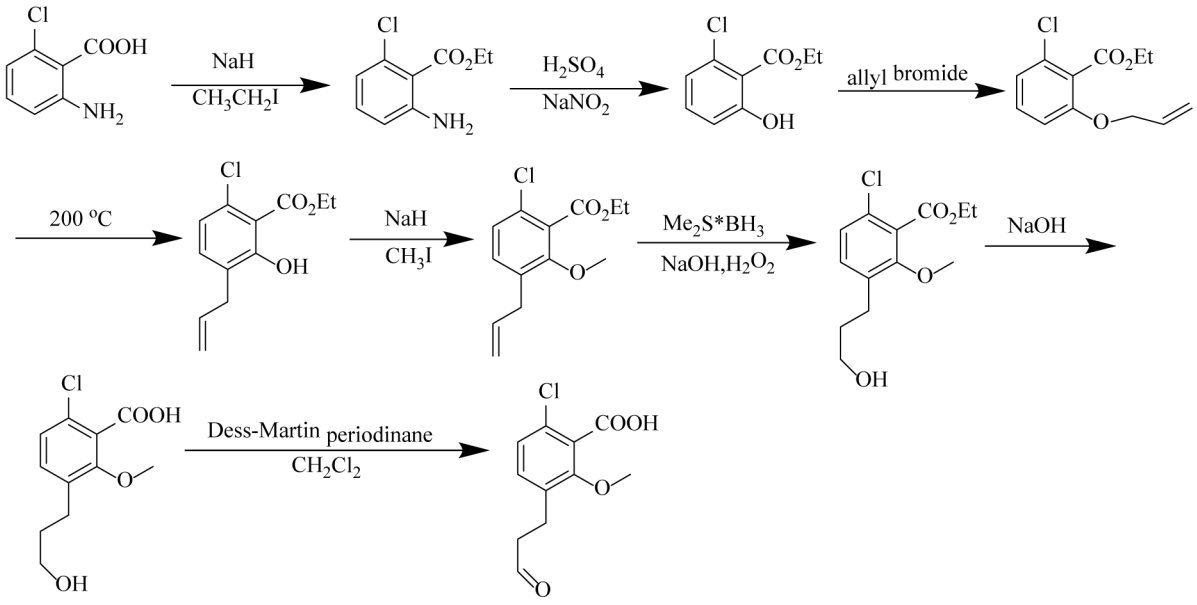
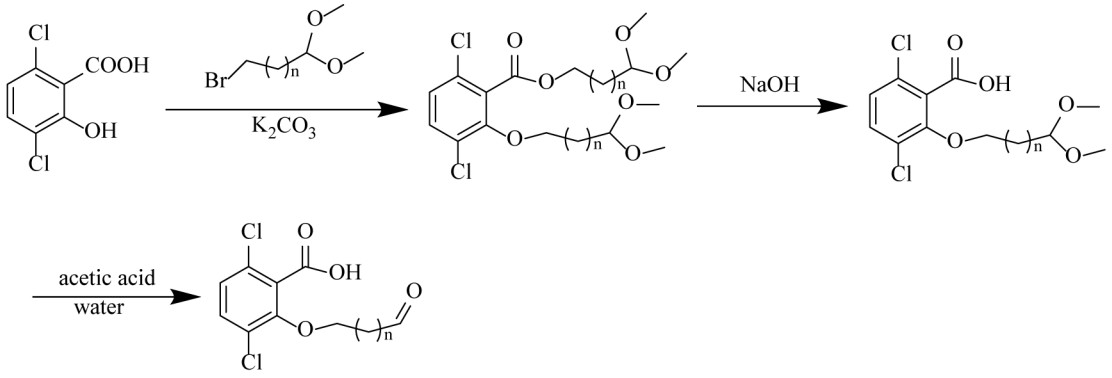


Figure 6.
The effect of soybean and soil matrix on the performance of antiserum #1000 based CLEIA.



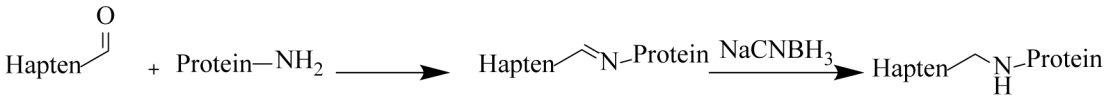
Hapten JQ-00-21



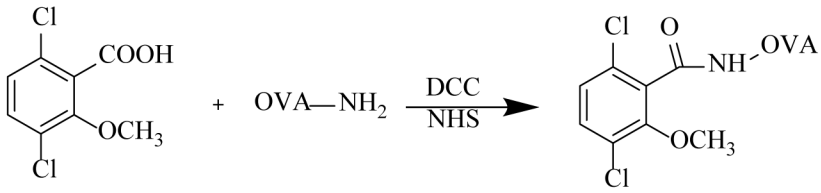
n=1 Hapten JQ-00-25
n=3 Hapten JQ-00-24

Scheme 1.
Synthetic route for dicamba haptens

Reductive amination method



Direct coupling



Scheme 2.

Preparation of hapten-protein conjugates. Protein = BSA, OVA, Thy.

Table 1.

Some reported assays for the determination of dicamba

Number	Detection methods	IC_{50}	LOD or LOQ	Samples	Reference
1	LC-MS/MS	-	0.126 ng/g	soil	(11) Xiong, et al.
2	Positive ESI LC-MS/MS	-	1.0 to 3.0 mg/kg	raw agricultural commodities	(12) Guo, et al.
3	HPLC-UV	-	0.2 μg/g	soil	(13) Voos, et al.
4	CE-UV	-	3.0 ng/mL	water	(14) Hadi, et al.
5	HPLC-UVD	-	6.0 μg/kg	food crops	(15) Shin, et al.
6	Immunoassays, polyclone antibody	195 μg/L	2.3 μg/L	water	(19) Clegg, et al.

Table 2.

Antiserum titer response against various coating antigens

Coat antigen (dilution 1000 fold)	JQ-00-21-Thy				JQ-00-24-Thy				JQ-00-25-Thy			
	# 997		# 998		# 999		# 1000		# 1001		# 1002	
	Dilution fold		Dilution fold		Dilution fold		Dilution fold		Dilution fold		Dilution fold	
	1000	10000	1000	10000	1000	10000	1000	10000	1000	10000	1000	10000
JQ-00-21-BSA	+++	+++	+++	+++	+++	+	+++	+	−	−	−	−
JQ-00-21-OVA	+++	+++	+++	+++	+++	+	+++	+	−	−	−	−
JQ-00-24-BSA	+++	++	+++	+++	+++	+++	+++	+++	−	−	−	−
JQ-00-24-OVA	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−	−
JQ-00-25-BSA	−	−	−	−	++	−	++	−	+++	+++	+++	+++
JQ-00-25-OVA	−	−	−	−	++	−	++	−	+++	+++	+++	+++
JQ-00-26-OVA	−	−	−	−	−	−	−	−	−	−	−	−

Note: −, absorbance < 0.3; +, absorbance 0.3-0.6; ++, absorbance 0.6-0.9; and +++, absorbance > 0.9

Table 3.

8 point competitive ELISA results for the best serum/coating antigen pairs (A: maximum absorbance; B: minimun absorbance; C: IC_{50} (ng/mL))

rabbit	immunogen	coating antigen	dilution		curve parameters		
			coat. antigen (μg/mL)	antiserum	A	B	C
998	JQ-00-21-Thy	24-BSA	0.5	1/8000	0.99	0.15	23.0
998	JQ-00-21-Thy	24-OVA	0.5	1/8000	1.13	0.29	18.4
999	JQ-00-24-Thy	25-BSA	0.5	1/8000	0.96	0.09	19.6
1000	JQ-00-24-Thy	24-BSA	0.5	1/80000	1.87	0.85	220.8
1000	JQ-00-24-Thy	21-OVA	0.5	1/8000	0.85	0.10	12.3
1000	JQ-00-24-Thy	25-BSA	5.0	¼000	1.15	0.24	23.1
1000	JQ-00-24-Thy	25-OVA	5.0	¼000	1.26	0.21	40.5

Table 4.

Cross-reactivity of the antiserum #998 and #1000 against dicamba structural analogs

Compound	Cross Reactivity (%)	
	#998	#1000
dicamba	100	100
5-hydroxydicamba	<0.1	<0.1
2,3,5-trichlorobenzoic acid	<0.1	<0.1
2,3,6-trichlorobenzoic acid	55	33
clopyralid	<0.1	<0.1
picloram	<0.1	<0.1
chloramben	<0.1	<0.1
chlorfenac	<0.1	<0.1

Table 5.

Spike-recovery results for soybean plant and soil samples determined by CLEIA and LC-MS. Antibody #1000 was used to analyze the spiked samples.

Sample	Spiked concentration (ng/mL)	CLEIA (ng/mL)	Average recovery (%)	CV (%)	LC-MS (ng/mL)	Average recovery (%)	CV (%)
	20	21.69±2.40	108	11.07	23.37±2.42	117	10.36
soybean plant	50	49.07±5.03	98	10.26	49.57±2.48	99	5.00
	150	128.80±10.58	86	8.22	113.7±7.22	76	6.35
	5	5.34±0.094	107	8.78	5.35±0.34	107	6.27
soil	15	15.7±0.09	105	8.62	17.4±0.51	116	2.95
	45	47.64±0.086	106	8.17	49.19±2.93	109	5.96

Table 6.

Quantification of dicamba in the real samples of soybean plant and soil determined by CLEIA and LC-MS. Antibody #1000 was used to analyze these samples.

Sample	#	Treatment dose, g/ha	Collection time, days after treatment	CLEIA (ng/g)	CV (%)	LC-MS (ng/g)	CV (%)
soybean plant	1	56	1	1043.95±64.13	6.14	997.92±84.86	8.50
	2	5.6	1	27.45±1.91	6.97	28.67±0.56	1.95
	3	0.56	1	4.76±0.17	3.66	4.66±0.26	5.64
	4	56	7	56.95±7.42	13.02	64.17±3.55	5.54
	5	56	14	177.81±8.54	4.81	179.79±6.74	3.75
	6	56	21	109.22±7.35	6.73	109.79±10.72	9.77
	7	56	39	ND	ND	2.35±0.19	7.97
	8	56	67	ND	ND	2.63±0.26	9.93
soil	1	56	1	165.68±2.79	1.68	168.25±12.8	7.61
	2	5.6	1	6.67±0.46	6.96	6.87±0.37	5.40
	3	0.56	1	ND	ND	0.80±0.17	21.48
	4	56	7	86.63±13.64	15.74	84.25±1.89	2.24
	5	56	14	19.8±1.42	7.19	17.87±0.77	4.32
	6	56	21	2.58±0.50	19.29	3.07±0.11	3.68