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Study on the Metabolism of Six Systemic Insecticides in a Newly Established Cell Suspension Culture Derived from Tea (Camellia Sinensis L.) Leaves

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Dear Editor,

Please find enclosed our manuscript entitled "***Study on the six systemic insecticides metabolism in a newly established cell suspension culture derived from tea (Camellia sinensis L.) leaves***" that we would like to be considered for publication in Journal of Visualized Experiments. This paper highlights a protocol for the establishment of a new tea cell suspension culture which was then served as a platform to study the metabolism behavior of six systemic insecticides. We consider of value publishing these data in Journal of Visualized Experiments, as they describe the establishment of a homogenous tea cell suspension culture from a loose tea callus derived of the sterile tea plantlets. The tea cell suspension was much simpler with no external interference which metabolites were easily obtained by comparison with the intact plant metabolism. Seven and two metabolites were found from thiamethoxam and dimethoate treated cell culture respectively, against two and only one similar metabolite from thiamethoxam and dimethoate treated intact tea plant. The techniques presented in this paper and demonstrated in video format will be highly useful for researchers working in the field of pesticides metabolism in other plants as well as the cell suspension culture.

Ruyan Hou designed the procedures described in the manuscript. *Weiting Jiao and Guoqin Ge* performed the experiments and analyzed the data. Finally, *Weiting Jiao* wrote the manuscript.

During the preparation and submission of this manuscript, we have been kindly assisted by Benjamin Werth.

Thank you for your consideration of this manuscript. We look forward to hearing from you.

Sincerely yours,

Ruyan Hou

TITLE:

Study on the Metabolism of Six Systemic Insecticides in a Newly Established Cell Suspension Culture Derived from Tea (*Camellia Sinensis* L.) Leaves

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KEYWORDS:

tea cell suspension, intact tea plant, insecticide, plant metabolism, metabolism, mass spectrometry

SUMMARY:

This work presents a protocol for establishing a cell suspension culture derived from tea (*Camellia sinensis* L.) leaves that can be used to study the metabolism of external compounds that can be taken up by the whole plant, such as insecticides.

ABSTRACT:

A platform for studying insecticide metabolism using in vitro tissues of tea plant was developed. Leaves from sterile tea plantlets were induced to form loose callus on Murashige and Skoog (MS) basal media with the plant hormones 2,4-dichlorophenoxyacetic acid (2,4-D, 1.0 mg L^{-1}) and kinetin (KT, 0.1 mg L^{-1}). Callus formed after 3 or 4 rounds of subculturing, each lasting 28 days. Loose callus (about 3 g) was then inoculated into B5 liquid media containing the same plant hormones and was cultured in a shaking incubator (120 rpm) in the dark at 25 ± 1 °C. After 3–4 subcultures, a cell suspension derived from tea leaf was established at a subculture ratio ranging between 1:1 and 1:2 (suspension mother liquid: fresh medium). Using this platform, six insecticides (5 µg mL^{-1} each thiamethoxam, imidacloprid, acetamiprid, imidaclothiz, dimethoate, and omethoate) were added into the tea leaf-derived cell

suspension culture. The metabolism of the insecticides was tracked using liquid chromatography and gas chromatography. To validate the usefulness of the tea cell suspension culture, the metabolites of thiamethoxan and dimethoate present in treated cell cultures and intact plants were compared using mass spectrometry. In treated tea cell cultures, seven metabolites of thiamethoxan and two metabolites of dimethoate were found, while in treated intact plants, only two metabolites of thiamethoxam and one of dimethoate were found. The use of a cell suspension simplified the metabolic analysis compared to the use of intact tea plants, especially for a difficult matrix such as tea.

INTRODUCTION:

Tea is one of the most widely consumed non-alcoholic beverages in the world[1,2]. Tea is produced from the leaves and buds of the woody perennial *Camellia sinensis* L. Tea plants are grown in vast plantations and are susceptible to numerous insect pests[3,4]. Organophosphorus and neonicotinoid insecticides are often used as systemic insecticides[5] to protect tea plants from pests such as whiteflies, leaf hoppers, and some lepidopteran species[6,7]. After application, these insecticides are absorbed or translocated into the plant. Within the plant, these systemic insecticides may be transformed through hydrolysis, oxidation or reduction reactions by plant enzymes. These transformation products can be more polar and less toxic than the parent compounds. However, for some organophosphates, the bioactivities of some products are higher. For example, acephate is metabolized into the more toxic methamidophos[8,9], and dimethoate into omethoate[10,11]. Plant metabolic studies are thus important for determining the fate of a pesticide within a plant[12].

Plant tissue cultures have been proven to be a useful platform for investigating the pesticide metabolism, with the identified metabolites similar to those found in intact plants[13-15]. The use of tissue cultures, particularly cell suspension cultures, has several advantages. Firstly, experiments can be carried out free of microorganisms, thus avoiding the interference of pesticide transformation or degradation by microbes. Secondly, tissue culture provides consistent materials for use at any time. Thirdly, the metabolites are easier to extract from tissue cultures than from intact plants, and tissue cultures often have fewer interring compounds and lower complexity of compounds. Finally, tissue cultures can more easily be used to compare a series of pesticides metabolism in a single experiment[16].

In this study, a cell suspension derived from the leaves of sterile-grown tea plantlet was successfully established. The tea cell suspension culture was then used to compare the dissipation behaviors of six systemic insecticides.

This detailed protocol is intended to provide some guidance so that researchers can establish a plant tissue culture platform useful for studying the metabolic fate of xenobiotics in tea.

PROTOCOL:

1. Tea callus culture

NOTE: Sterile leaves were derived from in vitro-grown plantlet lines first developed in the research group[17]. All procedures up to section 5 were carried out in a sterile laminar flow hood, except for the culture time in an incubator.

1.1. Adjust the pH of the two media (Murashige and Skoog [MS] basal medium and Gamborg's B5 liquid medium) to 5.8 prior to autoclaving (121 °C, 20 min).

1.2. Cut along the middle vein of a sterile leaf using scissors, and then subdivide each half into small pieces of about 0.3 cm x 0.3 cm in a petri dish.

1.3. Place the sterile explants (the small leaf pieces) onto MS basal media containing the plant hormones 2,4-D (1.0 mg L^{-1}) and KT (0.1 mg L^{-1}). Six explants can be placed in a 300 mL flask containing 100 mL of MS basal media.

1.4. Culture the above leaf explants at a constant temperature of 25 °C in the dark. After 28 days, select the first generation of induced callus and transfer to fresh flask (a subculture). Acquire the loose and friable callus after 3−4 subcultures.

2. Tea cell suspension culture

2.1. Cut the vigorous, friable and loose calluses from the solid medium into small pieces (range here 0.5−2 mm) using a sterile surgical blade under sterile conditions.

2.2. Weigh about 3 g of the small pieces of callus. Place the callus into a 150 mL flask containing 20 mL of B5 liquid media supplemented with 2,4-D (1.0 mg L^{-1}) and KT (0.1 mg L^{-1}).

2.3. Culture the liquid cell suspension at a constant temperature (25 ± 1 °C) in a shaking incubator at 120 rpm in the dark.

2.4. After 7 to 10 days of culturing, remove the culture flasks and let them stand for a few minutes.

2.5. Take all the supernatant as seed material for subculture to fresh medium (subculture ratio of suspension mother liquid to fresh medium ranged between 1:1 and 1:2). Remove the precipitated, large calluses.

2.6. Obtain the final well-grown cell suspension culture after 3−4 subculture cycles of 28 days each.

3. Triphenyl tetrazolium chloride assay of cell viability

3.1. Kill a sample of living cells at 100 °C for 10 min as a control cell before viability staining.

3.2. Centrifuge all cell suspension culture for 8 min at 6000 x *g*. Remove the supernatant

before suspending the cells in 2.5 mL of phosphate-buffered saline (PBS) buffer (pH 7.3), and shake it for 1 min by hand.

3.3. Add 2.5 mL of the 0.4% triphenyl tetrazolium chloride (TTC) solution and shake by hand again.

3.4. Incubate the mixture for 1 h in a standing incubator (30 °C).

4. Treatment and sampling of tea cell suspension cultures with insecticides

4.1. Add an aliquot of 400 µL of filter-sterilized stock solution (500 µg mL^{-1}) of four neonicotinoids (thiamethoxam, acetamiprid, imidacloprid, and imidaclothiz) or two organophosphates (dimethoate and omethoate) into the cell suspension cultures, respectively.

NOTE: If the aim is to compare xenobiotic behaviors, use the same mother batch of cell suspensions to test the different compounds.

4.2. Culture the samples of cell suspensions with insecticides at constant temperature (25 ± 1 °C) and shaking incubator speed (120 rpm). Take the samples (see step 4.3 or 4.4) on 0, 3, 5 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75 days.

4.3. To test a sample containing a neonicotinoid, remove a 1 mL aliquot of the homogeneous cell culture, place it into a 1.5 mL plastic centrifuge tube, and centrifuge at 4000 x *g* for 2 min.

4.3.1. Pass the supernatants through a 0.22-µm pore-size filter membrane before analysis by high-performance liquid chromatography-ultraviolet (HPLC-UV) and ultra-high performance liquid chromatography-quadrupole time-of-flight (UPLC-QTOF) mass spectrometry (**Table of Materials**).

4.4. To test a sample containing an organophosphate, remove a 500 µL aliquot of the cell culture and place into a 35 mL centrifuge tube or a 1.5 mL plastic centrifuge tube (prepare the latter sample like that of neonicotinoid).

4.4.1. Add 0.1 g of sodium chloride and 5 mL of acetone/ethyl acetate (3:7, v/v) into the 35 mL centrifuge tube of the 500 µL samples.

4.4.2. Vortex the mixtures for 1 min, and then allow them to rest for 10 min.

4.4.3. Take 2.5 mL of the supernatant into a 10 mL glass tube and evaporate to near-dryness using a nitrogen evaporator at 40 °C.

4.4.4. Dissolve the residue with 1 mL acetone, vortex for 1 min, pass it through a 0.22-µm filter membrane before analysis by gas chromatography–flame photometric detector (GC-

FPD).

5. Sample preparation of intact tea plant with insecticides

NOTE: The intact tea plant trial was conducted in a hydroponic system using tea seedlings grown in 50 mL of a nutrient solution (30 NH_4^+, 10 NO_3^-, 3.1 PO_4^-, 40 K^+, 20 Ca^{2+}, 25 Mg^{2+}, 0.35 Fe^{2+}, 0.1 B^{3+}, 1.0 Mn^{2+}, 0.1 Zn^{2+}, 0.025 Cu^{2+}, 0.05 Mo^+, and 10 Al^{3+}, in mg L^{-1}) [18]. An experimental greenhouse was under a light-dark cycle (12 h of light and 12 h of darkness) at 20 °C at Anhui Agricultural University.

5.1. Put five plants in a 4 L plastic pot for 15 days.

5.2. Add 0 ppm (control) or 100 ppm of thiamethoxam or dimethoate into plastic pots, respectively.

5.3. Prepare the intact plant sample according to the previous method, except for presoaking[19], and then analyze with mass spectrometry for an accurate mass spectrum.

6. Instrument analysis

6.1. HPLC analysis of the metabolic behavior of neonicotinoids

6.1.1 Use an HPLC-UV (**Table of Materials**) to detect the content and metabolic products of thiamethoxam and acetamiprid at a wavelength of 254 nm, and of imidacloprid and imidaclothiz at 270 nm in samples from sections 4.3 and 4.4.

NOTE: The HPLC-UV condition was the same as the previous study[19].

6.2. GC analysis of the metabolic behavior of organophosphates

6.2.1. Detect the content of dimethoate and omethoate in samples from sections 4.5 to 4.9 by a GC-FPD using a chiral column (**Table of Materials**).

6.2.2. Use nitrogen as the carrier gas and set the flow rate at 1.0 mL min^{-1}.

6.2.3. Set the initial temperature to 120 °C, and hold it for 5 min. Increase the temperature to 150 °C at 30 °C min^{-1} and hold for 3 min. Increase to 170 °C at 10 °C min^{-1} and hold for 7 min. Finally increase to 210 °C at 30 °C min^{-1} and then hold for 5 min.

6.2.4. Set the injection temperature to 200 °C in splitless mode; Set the detector temperature to 250 °C.

6.2.5. Set the injection volume to 1 µL.

6.3. UPLC-QTOF analysis of the insecticide metabolites in cell culture

6.3.1. Detect the metabolites of the insecticides in cell culture (samples from sections 4.3 and 4.4) using UPLC-QTOF with a C18 column (**Table of Materials**).

6.3.2. Set the flow rate to 0.2 mL min^{-1}. Set the injection volume to 10 µL.

6.3.3. For the neonicotinoid-treated samples, set the initial mobile phase to 85% A (5 mM ammonium formate water) and 15% B (acetonitrile). Over 10 min, increase mobile phase B to 38% and return to 15% over 1 min, hold for 9 min.

6.3.4. For the organophosphate-treated samples, set the initial mobile phase to 55% A (0.1% formic acid water) and 45% B (acetonitrile). Over 5 min, increase mobile phase B to 70%, then return to 45% of B over 0.5 min, hold for 2.5 min.

6.3.5. Set the QTOF operation parameters as follows: gas temperature, 325 °C; drying gas (nitrogen), 10 L min^{-1}; sheath gas temperature, 350 °C; sheath gas flow, 11 L min^{-1}; capillary voltage, 4000 V; nozzle voltage, 1000 V; fragmentor voltage, 100 V for neonicotinoid insecticides or 110 V for organophosphorus insecticides; skimmer voltage, 65 V; operating in positive ion mode.

6.3.6. Set the instrument to the full scan spectrum and target MS/MS mode.

6.3.7. Process the data using accurate mass tools; Infer the metabolites with no standard products from the MS/MS annotation as well as the literature[12,15,20-22].

6.4. UPLC-Orbitrap analysis of the insecticide metabolites in intact plant extract

6.4.1. Detect the metabolites of insecticides in intact plant extract using UPLC-Orbitrap mass spectrometry (**Table of Materials**).

6.4.2. Set the mass spectrometry (**Table of Materials**) operation parameters as follows: sheath gas pressure, 35 arb; gas temperature, 300 °C; nozzle voltage, 3.5 KV; capillary temperature, 350 °C.

6.4.3. Set the elution programs as the above (steps 6.3.3 and 6.3.4) for UPLC-QTOF analysis of cell culture.

REPRESENTATIVE RESULTS:

The induction of callus from leaves harvested from field-grown tea trees and from leaves excised from tea plantlets grown in vitro in a sterile environment was compared by measuring contamination, browning, and induction after 28 days of cultivation on MS media (**Figure 1A**). Callus growth was recorded at 20, 37, 62 and 90 days of culture (**Figure 1B**). The callus derived from the in vitro-grown leaves showed more vigorous growth than did the callus derived from

the field-grown leaves during the whole 90 days of cultivation. The callus from the sterile leaves was bright yellow, while the callus from the field-grown leaves was brown (**Figure 1B**).

At a concentration of 1.0 mg L^{-1} of 2,4-D[23], the concentration of KT was optimized. At 0.05 mg L^{-1} KT, the callus growth rate was slow, the texture was a little compact, and the callus was white in color (**Figure 2C**); at 0.1 mg L^{-1} KT concentration, the callus growth rate was the highest, up to 61.5% (**Figure 2A**), the texture was loose, and the color was yellowish (**Figure 2C**); when the KT was increased to 0.5 mg L^{-1}, the callus was compact and irregular and brown in the center (**Figure 2C**). After the KT concentration was selected, the concentration of 2,4-D was further studied. At a combination of 1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} KT, the callus growth rate was the highest, reaching 46.9%, and the appearance of the callus was the best (**Figure 2B,D**).

After the 2nd subculture on solid media, more than half of the surface of each excised leaf was covered by growing callus (**Figure 3A**). After the 4th subculture, the leaf sections were completely covered by the callus. After the 5th subculture, the callus texture began to become compact with some white and brown spots on the bottom.

When the subculture cycle was 21 days long (**Figure 3B**), the callus was vigorous, but the greatest amount of growth had not been reached, indicating that frequent subculture would result in less callus amount. When the subculture cycle was 28 days long, the callus had grown vigorously, the color was yellowish color and the texture was loose. After 35 days, the callus began to brown from the center. The callus was in the worst state, with a deep brown color and no longer growing, at 42 days.

Two kinds of liquid media were compared for their effects on the growth of the callus and the color of the cell suspension (**Figure 4**). Three different ratios of mother liquid to total volume of culture liquid were tested. During the 75 days of cultivation, the cell density gradually increased in cultures started at all three ratios. The ratio of 15 g cells in 40 mL fresh media (v/v) yielded an optical density (OD) value significantly higher than that of 4 g in 40 mL (v/v) and 6 g in 40 mL (v/v) (**Figure 5A**). After 4 subculture cycles of 28 days each, a tea cell suspension system was successfully established from sterile tea callus in B5 liquid media (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Comparison of callus induction from picked leaves and sterile plantlet leaves. (**A**) Comparison of callus induction from leaves harvested from plantation-grown tea plants and from leaves excised from sterile, in vitro-grown plantlets. Explants were observed for contamination, browning and induction of callus. (**B**) Comparison of callus growth of leaves derived from plantation-grown tea plants (set 1) and sterile in vitro-grown plantlets (set 2): Photographs were on different days: 20 (panels **a**); 37 (**b**); 62 (**c**); and 90 (**d**). This figure has been modified from Jiao et al.[24].

Figure 2: The growth rate and growth status of tea-leaf derived callus under different plant hormone concentrations. The growth rate (**A**) and growth status (**C**) of tea-leaf derived callus under different KT concentrations and 1 mg L^{-1} 2,4-D; The growth rate (**B**) and growth status (**D**) of callus under different 2,4-D concentrations and 0.5 mg L^{-1} KT. This figure has been modified from Jiao et al.[24].

Figure 3: Callus status after different numbers of subculture cycles (A) and different lengths of subculture cycles (B). This figure has been modified from Jiao et al.[24].

Figure 4: Influence of different media types on callus growth in liquid suspension culture system. (**A**) B5 medium. (**B**) MS medium. This figure has been modified from Jiao et al.[24].

Figure 5: The optical density values and TTC staining. (**A**) The OD_{680} value of cell suspension started at different ratios from 0 to 75 days; (**B**) The TTC staining of living and control cells. This figure has been modified from Jiao et al.[24].

Figure 6: The process of establishing a tea cell suspension culture at constant temperature (25 ± 1 °C) in a dark incubator. Sterile culture of tea plantlets as source of leaf explant (**a**); Tea leaf inoculated onto MS medium with 2,4-D (1.0 mg L^{-1}) and KT (0.1 mg L^{-1}) (**b**); Initial cultured callus after 28 days (**c**); Callus suitable for cell suspension after 4 subculture cycles of 28 days each (**d**); The remaining steps were at the same temperature but at a constant speed of 120 rpm in a shaking incubator: Callus inoculated into B5 medium for 7 to 10 days (**e**); Seeded cell suspension after removing the precipitated and large callus (**f**); The subculture of cell suspension after 1 cycle of 28 days (**g**); Mature cell suspension after 3-4 subculture cycles of 28 days each (**h**). This figure has been modified from Jiao et al.[24].

Supplemental Figure 1: The metabolism of 5 µg/mL of 6 insecticides in tea cell suspension culture and in media (CK) incubated at constant temperature (25 ± 1 °C) and shaking incubator speed (120 rpm) over 75 days. Thiamethoxan (**A**), imidacloprid (**B**), acetamiprid (**C**), imidaclothiz (**D**), dimethoate (**E1**), and omethoate (**F**); (**E2**) Production over time of the metabolite of dimethoate (omethoate) produced in dimethoate-treated cell culture and media. This figure has been modified from Jiao et al.[24].

Supplemental Figure 2: Total ion chromatograms (TICs) of the extracts from untreated control cell culture, thiamethoxam-treated cell culture, thiamethoxam-treated media (cell-free) after 75 days. Peaks 1-5, 7 and 8 were metabolites of thiamethoxam and Peak 6 was thiamethoxam (**A**); TICs of the extracts from dimethoate-treated cell culture, dimethoate-treated media (cell-free), and untreated control cells after 60 days. Peaks 1 and 2 were metabolites of dimethoate and Peak 3 was dimethoate (**B**); TICs of the extracts from thiamethoxam-treated (upper) and untreated (lower) intact plants (**C**); TICs of the extracts from dimethoate-treated (upper) and untreated (lower) intact plants (**D**); The metabolite of dimethoate at *tR* 1.86 min in intact plants (D1); No compounds detected at *tR* 1.86 min in untreated plants (D2). This figure has been modified from Jiao et al.[24].

Supplemental Figure 3: The secondary mass spectrometry using UPLC-QTOF of peaks derived from cultures treated with (A) thiamethoxam and (B) dimethoate. This figure has been modified from Jiao et al.[24].

Supplemental Figure 4: The secondary mass spectrometry using Q-Exactive of peaks derived from intact plant treated with thiamethoxam (A1, A2 and **A3) and dimethoate (B1** and **B2).** This figure has been modified from Jiao et al.[24].

DISCUSSION:

This article presents the detailed process of establishing a model of pesticide metabolism in tea plant tissue, including the selection of explants, the determination of cell viability, and the establishment of a tea cell suspension culture with high metabolic activity. Any parts of a plant tissue could be used to initiate callus in a sterilized environment[25]. Tea leaves were chosen for callus initiation in this study, not only because leaves to tend to be less contaminated than the parts below ground, but also because they are the edible part of the crop and the main target of pesticide application.

In this study, the induction rate and growth status of callus from leaves picked from the field and from leaves excised from a sterile plantlet grown in vitro were compared. The sterile leaves had much lower rates of browning and contamination and a higher rate of induction compared to field-grown leaves. This was likely because leaves from field-grown plants not only underwent surface sterilization using ethanol and mercury but also a change in growth environment, while sterile leaves were cultivated in a sterile environment and could be used directly without additional sterilization. In addition, the callus derived from the in vitro-grown, sterile plantlets showed more vigorous growth than the field-grown leaves during the 90 days of cultivation. Leaves from sterile plantlets were more suitable for induction of tea callus, not only because of their high callus induction and low contamination rates, but also because of the shorter pre-treatment time and independence from seasonal factors.

To culture loose and friable callus, the crucial parameters, primarily plant growth regulator levels and length of and number of subculture cycles, must be optimized[25]. 2,4-D can effectively promote callus induction and growth and is the most widely used hormone in callus culture[26]. Subculture times and subculture cycle length are also important for callus culture[25]. After 2 to 4 subcultures of 28 days of each cycle, the callus had a loose texture with a yellowish color and no browning. The optimization experiments determined that the best callus induction protocol was to place leaf explants from sterile plantlets on MS basal media containing 1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} KT and to transfer the explant/callus every 28 days for a total of 4 subculture cycles. This protocol initiated loose and friable callus that was suitable for the initiation of a cell suspension.

In plant tissue culture, the solid medium used for callus growth can often be used for cell suspension culture in a liquid form[23]. Whereas, tea comes into being large quantities of polyphenols in MS basal medium containing a high concentration of inorganic salts, resulting in the callus browning[27,28]. In this study, liquid B5 media and MS media were both tested. The

average growth rates were found no significantly difference between the two cultures (16.66% in B5 basal media and 15.77% in MS basal media; **Figure 4**). However, the calluses were brown in MS basal media. So, B5 basal media was selected in the proposed method.

Oxygen is important to plant cell growth and metabolism. In liquid culture, an excessive volume of liquid will decrease the oxygen concentration and inhibit cell growth, while too little liquid also inhibits cell growth[25]. Several ratios of liquid to flask volume (mL liquid: mL flask) were tested. Based on the dry weight of the cell growth after 21 d, the liquid: flask ratios ranked as follows: 30:150 > 40:150 > 20:150, 50:150, 60:150 (mL: mL)[23]. In this study, 40 mL of culture liquid was placed in a 150 mL flask (40 mL: 150 mL) was selected according to the how the cell suspension looked as observed by the naked eye.

Plant cells cannot grow well when the cell density is too high or too low. Thus, the proportion of mother cell suspension culture to fresh medium at the time of subculture affects the growth potential of the cells[29]. This study used the OD value of the homogeneous cell suspension culture to represent the amount of cell growth. Inoculation with 15 g of mother liquid into 40 mL of total volume of culture liquid (v/v) was suitable for the cell growth. The subculture ratio was equal to between 1:1 and 1:2 (suspension mother liquid: fresh medium). Cell viability within the tea cell suspension culture was tested by TTC staining. The colorless TTC compound can be converted to the red colored formazan by dehydrogenases in the mitochondria of living cells, but the color cannot be changed from dead cells (**Figure 5B**). This method verified the growth status of the cells in liquid culture.

The establishment of a tea cell suspension culture provides an easy in vitro research platform for studying metabolism and metabolites of different pesticides. Independent of season and weather, cell suspension cultures can be treated with different pesticides, different concentrations of active ingredient, and for different lengths of time. The metabolites produced in the tea cell suspension cultures were similar to those extracted from intact plants (**Supplemental Figure 1** and **Supplemental Figure 2**). Interestingly, seven metabolites of thiamethoxam and two metabolites of dimethoate were detected in tea cell suspension culture, but only two metabolites of thiamethoxam and one for dimethoate in treated intact plants (**Supplemental Figure 1**, **Supplemental Figure 2**, and **Supplemental Figure 3**). This may be because of the easier extraction from cells without waxy cuticle, fewer compounds from the tea interfering with the mass spectrometry results (the matrix effect), or the simpler metabolite profile of tea cells compared to the whole plant.

The results showed that thiamethoxam was more readily metabolized by the tea cell compared with the other three neonicotinoids (**Supplemental Figure 4**). Both of the organophosphates (dimethoate and omethoate) were metabolized faster than the neonicotinoids. These results show the diversity of the metabolic pathways and of metabolic regulation in the tea cell, which need to be further studied.

Using intact plants to study insecticide metabolism and to identify insecticide metabolites presents numerous difficulties, including barriers to absorption and long-distance transport

of both initial and breakdown compounds within the plant[30]. Cell suspension cultures could not only solve this problem, but also reduce matrix interference in sample analysis compared to the extract from fresh leaves[24]. This research proved that tea cell suspension cultures are an effective platform for studying the metabolism of xenobiotic compounds in the tea plant. It can be served as a mode to study the metabolism of xenobiotics in other plants.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1.

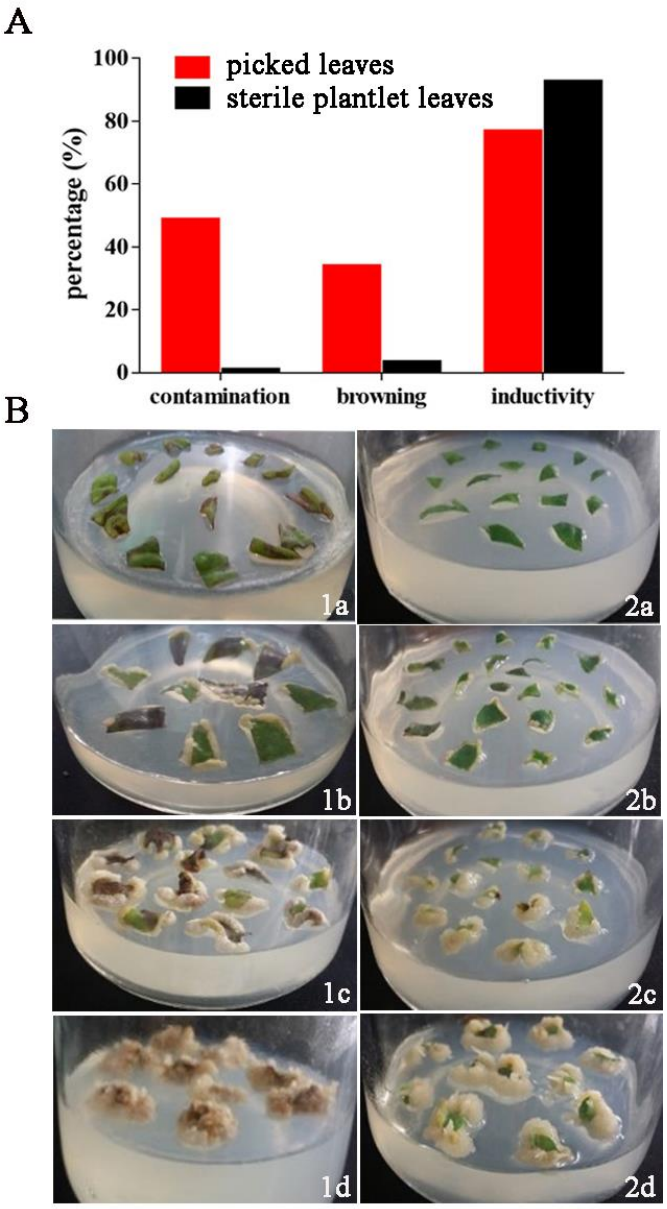


Figure 2.

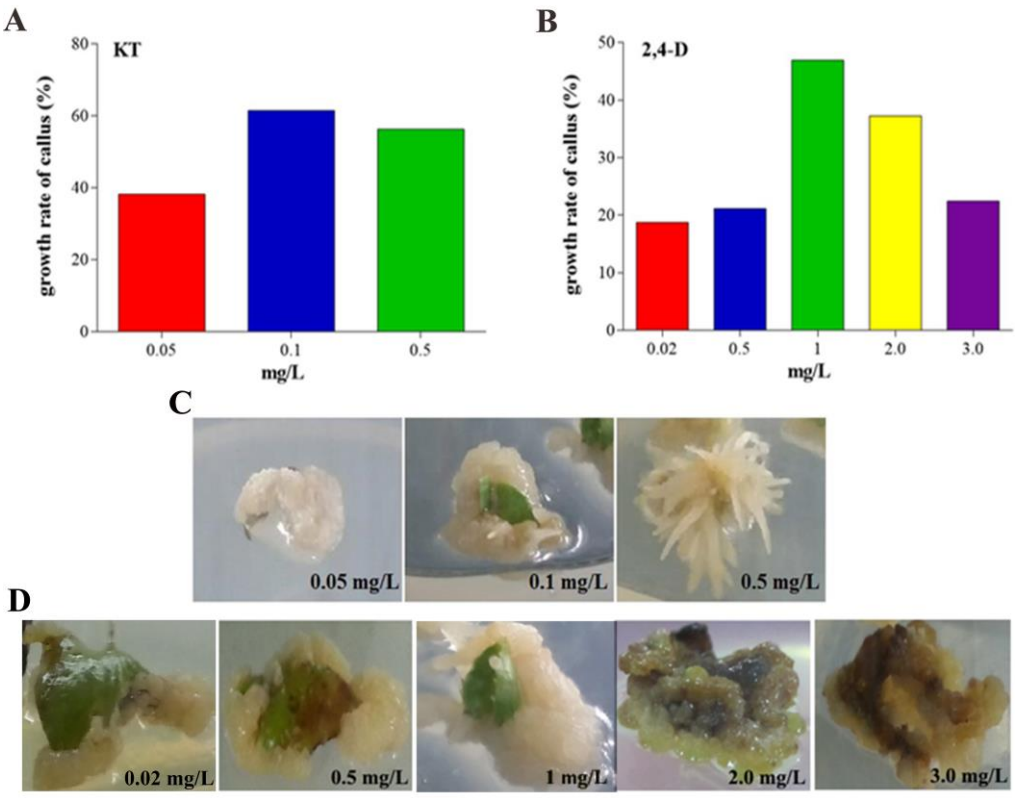


Figure 3.

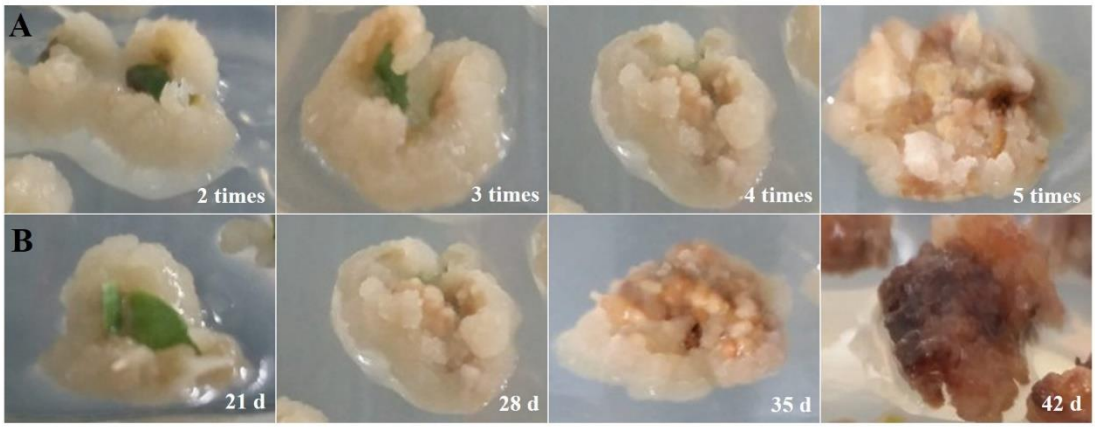


Figure 4.

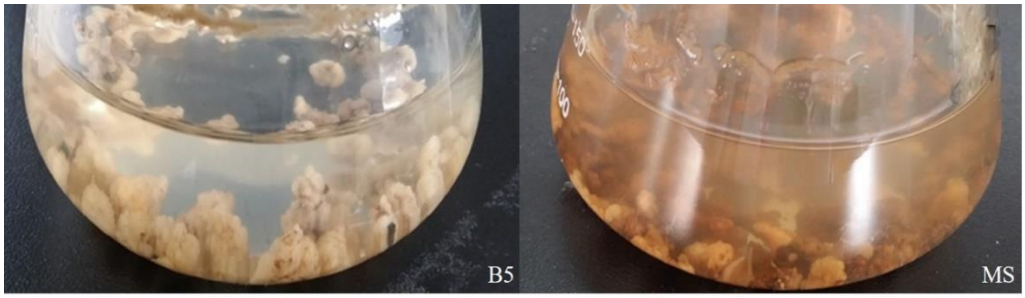


Figure 5.

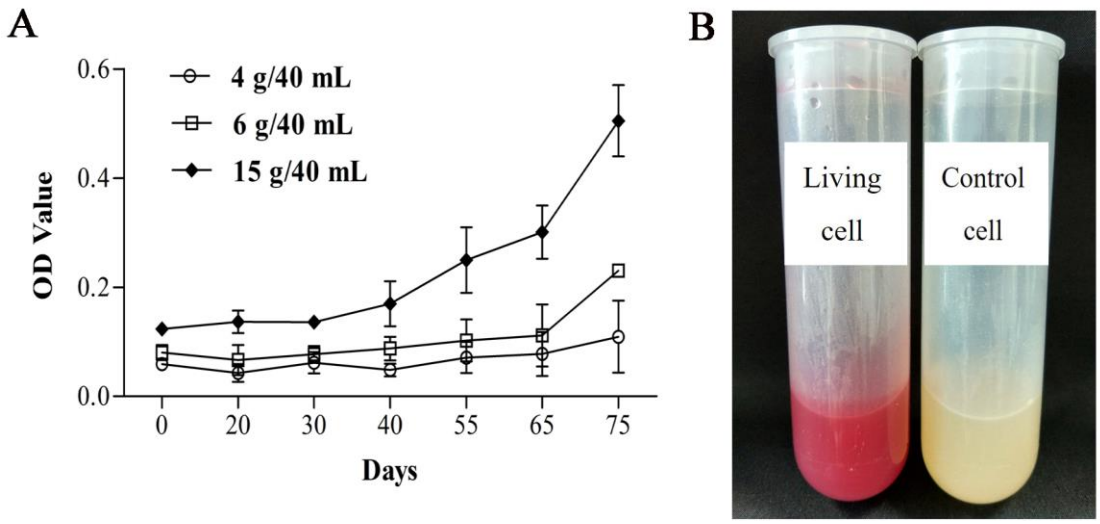
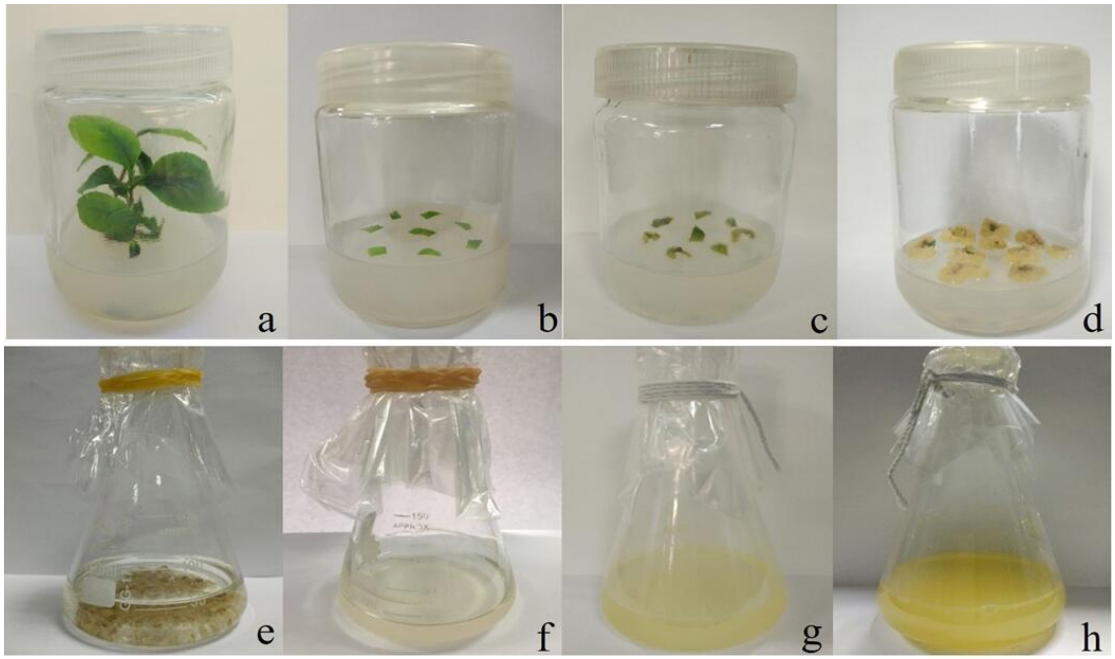


Figure 6.



Materials and Equipment

Name	Company	Catalog Number
Acetamiprid (99.8%)	Dr. Ehrenstorfer	46717
Acetonitrile (CAN, 99.9%)	Tedia	AS1122-801
Agar	Solarbio Science & Technology	A8190
Clothianidin (99.8%)	Dr. Ehrenstorfer	525
Dimethoate (98.5%)	Dr. Ehrenstorfer	109217
Imidacloprid (99.8%)	Dr. Ehrenstorfer	91029
Imidaclothiz (99.5%)	Toronto Research Chemical	I275000
Kinetin (KT, >98.0%)	Solarbio Science & Technology	K8010
Omethoate (98.5%)	Dr. Ehrenstorfer	105491
Polyvinylpolypyrrolidone (PVPP)	Solarbio Science & Technology	P8070
Sucrose	Tocris Bioscience	5511
Thiamethoxam (99.8%)	Dr. Ehrenstorfer	20625
Triphenyltetrazolium Chloride (TTC, 98.0%)	Solarbio Science & Technology	T8170
2,4-Dichlorophenoxyacetic Acid (2,4-D, >98.0%)	Guangzhou Saiguo Biotech	D8100
chiral column	Agilent CYCLOSIL-B	112-6632
Gas chromatography (GC)	Shimadu	2010-Plus
High-performance liquid chromatography (HPLC)	Agilent	1260
HSS T3 C18 column	Waters	186003539
Ultra-high-performance liquid chromatography (UPLC)	Agilent	1290-6545
Ultra-high-performance liquid chromatography (UPLC)	Thermo Scientific	Jltimate 3000-Q Exactive Focu

Comments
CAS No: 135410-20-7
CAS No: 75-05-8
CAS No: 9002-18-0
CAS No: 210880-92-5
CAS No: 60-51-5
CAS No: 138261-41-3
CAS No: 105843-36-5
CAS No: 525-79-1
CAS No: 1113-02-6
CAS No: 25249-54-1
CAS No: 57-50-1
CAS No: 153719-23-4
CAS No: 298-96-4
CAS No: 94-75-7
Chromatography column (30 m × 0.25 mm × 0.25 µm)
Paired with Flame Photometric Detector (FPD)
Paired with Ultraviolet detector (UV)
Chromatography column (100 mm × 2.1 mm × 1.8 µm)
Tandem quadrupole time-of-flight mass spectrometer (QTOF)
Connected to a Orbitrap mass spectrometer

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Author(s): Weiting Jiao#, Guoqin Ge#, Rimao Hua, Jun Sun, Yeyun Li, Ruyan Hou*

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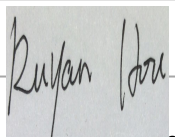
Signature:	
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Response: Thank you for your revision. The manuscript was carefully revised by us, including American editor Anita K. Snyder. And we have marked the revisions in yellow highlighting in the revised manuscript.

2. Please revise lines 70-72, 87-88, 231-234, 236-238, 248-250, 268-276, 278-281, 283-292, 303-304, 309-310, 322-324, 329-336, 344-346, 348-350, 359-365 to avoid previously published text.

Response: Thank you for your revision. The manuscript was carefully revised at line 70-73, 89-91, 244-246, 248-250, 260-262, 282-288, 289-291, 292-303, 339-340, 345-347, 358-360, 365-371, 379-381, 381-382, 401-405 in yellow highlighting.

3. Please define all abbreviations before use.

Response: Thank you for your revision. We have defined them in the revised manuscript in yellow highlighting. And all abbreviations are listed in the table of Materials and Equipment as required.

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Thank you for your revision. We have deleted the personal pronouns in the revised manuscript in yellow highlighting.

5. Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and with no more than 4 sentences), numbered header, or a 'Note'. Please move the introductory paragraphs (e.g., lines 101-105) of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.

Response: Thank you for your revision. We have moved the introductory paragraphs (lines 101-105) to line 109-114 in yellow highlighting.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

7. 1.1: What is used to cut? Is this done in a petri dish or container?

Response: Thank you for your revision. They have been supplemented in section 1.2 at line 116-117 in yellow highlighting.

8. 1.2: Do the explants refer to the small leaf pieces? What volume of media is used? What are 2,4-D and KT?

Response: Thank you for your revision. They have been supplemented in section 1.3 at line 119-121 in yellow highlighting. 2,4-D and KT are the plant hormones, and they have been defined at line 60 in yellow highlighting.

9. 1.3: It is not clear what 3-4 times of subculture number and 28 d lengths refer to. At what conditions are the leaves cultured? How to acquire the loose and friable callus?

Response: Thank you for your revision and question. They have been supplemented in section 1.4 at line 123-125 in yellow highlighting. 3-4 times of subculture number means that it need to subculture the callus for 3-4 times after the first-generation callus was produced; 28 d lengths refer to the length of each subculture-period. The loose and friable callus was acquired under the above condition, including the plant hormones and subculture times et al.

10. 2.1: Please define the size of small pieces.

Response: Thank you for your question. They have been supplemented at line 129-130 in yellow highlighting.

11. 2.2: What container is used? What volume of media is used?

Response: Thank you for your revision. They have been supplemented in section

2.2 at line 132-134 in yellow highlighting.

12. 2.3: Please change the unit “r min-1” to “rpm”.

Response: Thank you for your revision. It has been revised at line 137 in yellow highlighting.

13. 2.4: What volume of supernatant is used? How to obtain the supernatant?

Response: Thank you for your revision. They have been supplemented in section 2.4 and 2.5 at line 139-144 in yellow highlighting.

14. 3.1: What is defined as the control cell?

Response: Thank you for your question. We have revised the sentence at line 150.

15. 3.2: Please specify centrifugation conditions (centrifugal force in x g and time). What is the rotating/shaking speed?

Response: Thank you for your revision. They have been supplemented in section 3.2 at line 152-153 in yellow highlighting.

16. 4.1: Are these compounds added separately to each cell suspension or added as a mixture to one cell suspension?

Response: Thank you for your revision. These compounds were added separately to each cell suspension, and we have revised it at line 165 in yellow highlighting.

17. 4.2: Please specify the days of sampling.

Response: Thank you for your revision. They have been supplemented in section 4.2 at line 171-172 in yellow highlighting.

18. 5.1: Please provide the composition of nutrient solution. Please specify the size of the plastic pot. How many seedlings are planted in one pot?

Response: Thank you for your revision. They have been supplemented at line 197-203 in yellow highlighting.

19. 6.1.1, 6.2.1, 6.3.1: Please specify the sample preparation for HPLC-UV, GC and UPLC-QTOF analyses, or mention from which step these samples are prepared/obtained.

Response: Thank you for your revision. They have been supplemented in section 6.1.1, 6.2.1, 6.3.1 in yellow highlighting.

20. 6.1.1: Please specify the wavelengths that are measured.

Response: Thank you for your revision. They have been supplemented at line 216-217 in yellow highlighting.

21. 5.3, 6.2.1: Please remove commercial language (Q-Exactive, CYCLOSIL-B, etc.).

Response: Thank you for your revision. They have been revised at line 209 and 223 in yellow highlighting. And the specific information is shown in table of materials and equipment.

22. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Response: Thank you for your revision. Each picture has been uploaded as a pdf.

23. Figure 5: Please abbreviate liters to L to avoid confusion and include a space between numbers and their corresponding units (i.e., 4 g/40 mL, 6 g/40 mL, etc.). At what wavelength is the OD measured? In panel B, please identify living and control cells.

Response: Thank you for your revision. They have been revised in Figure 5. The wavelength has been supplemented at line 324. And the living and control cells have been identified in panel B.

24. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

Response: Thank you for your revision. They have been removed.

25. References: If there are six or more authors, list the first author and then “et al.”.

Response: Thank you for your revision. They have been revised thoroughly.

Changes to be made by the Author(s) regarding the video:

1. Please revise the video based on the updated manuscript.

Response: Thank you for your revision. They have been revised carefully.

2. 04:21: Please change the unit “r min-1” to “rpm”.

Response: Thank you for your revision. They have been changed at 04:47.

3. 06:26, etc.: Please avoid commercial language (Q-Exactive).

Response: Thank you for your revision. They have been revised at 06:55.

4. 08:49: Please abbreviate liters to L to avoid confusion and include a space between numbers and their corresponding units (i.e., 4 g/40 mL, 6 g/40 mL, etc.).

Response: Thank you for your revision. They have been revised at 09:28.

5. 0:00-0:03 - This opening animation should be removed from the video, since JoVE did not produce the content.

Response: Thank you for your revision. They have been removed.

6. 2:41 - This edit is a jump cut, which tends to have a jarring effect on the viewer. It should be smoothed out with a crossfade instead.

Response: Thank you for your revision. They have been revised.

7. Please upload a revised high-resolution video here: https://www.dropbox.com/request/esZ64Qm9tk9W3YlEK9hG

Response: Thank you so much. They have been uploaded.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a new report regarding the metabolism behavior of systemic insecticides in tea cell suspension. The video manuscript provides the detail guidance to reader of the same research filed. It could serve as a model to study the metabolism behavior of other xenobiotics in tea or in other plants. However, a minor revision is required, the points are shown as the following.

Minor Concerns:

1. Line 356-357, in Fig. 5B, there is no color change of different concentration of cells, please check it.

Response: Thank you for your revision. We have deleted it and revised it at Line 388-389.

2. Why did the author use the different mass spectrometer (UPLC-Q-Exactive) to analysis the metabolites of insecticides in intact plant extract?

Response: Thank you for your suggestion. The matrix interference in intact plant extract was more severe than that in cell suspension. It was difficult to capture the metabolites of insecticides in in intact plant extract, therefore we choose the UPLC-Q-Exactive, which was more accurate mass spectrometer than UPLC-QTOF.

3. Some grammar issues need to be modified, such as Line 359 "A tea cell suspension platform was established and provided a convenient in vitro research system for studying metabolism and metabolites of six insecticides"; Line 342-343 "the liquid: flask volume of 40 mL: 150 mL…"

Response: Thank you for your suggestion. The manuscript was carefully revised by us, including American editor Anita K. Snyder. And we have marked the revisions in yellow highlighting in the revised manuscript at line 390-391 and 374-375.

4. Line 342-343, The liquid: flask volume of 40 mL: 150 mL was selected according to the status of the cell suspension. So, in video manuscript, at 3.04 min, what was the volume of the supernatant liquid of seed material for subculture after 7 to 10 days of culturing? And what was the volume of fresh medium? It seems to be less than 40 mL in volume.

Response: Thank you for your question. Yes, the optimal volume ratio of subculture of suspension liquid: flask was 40 mL: 150 mL. In order to obtain the largest number of cells, and because of the short culture period (7 to 10 days), the

volume of seed material was 20 mL, and the volume of fresh medium was also 20 mL, which was in total 40 mL.

Reviewer #2:

Manuscript Summary:

1. Line no. 81 write from instead of through

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references. "Transformed through" is commonly used.

2. Line no. 102 write was instead of were

Response: Thank you for your revision. We have revised it at line 113 in yellow highlighting.

3. Line no. 109 write was instead of were

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references. "Were" is commonly used.

4. Line no. 126 write inoculates instead of inoculate

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references, "inoculate" was replaced by "place", we have revised it at line 132 in yellow highlighting.

5. Line no. 160 write of instead of on

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references, we have revised it at line 170 in yellow highlighting.

6. Line no. 205 write was instead of were

Response: Thank you for your revision. We have revised it at line 218 in yellow

highlighting.

7. Line no. 213 write of instead of to

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references, we have revised it at line 225 in yellow highlighting.

8. Line no. 246 write to the full scans instead of to full scans

Response: Thank you for your revision. We have revised it at line 257 in yellow highlighting.

9. Line no. 248 write onto instead of with

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references, we have revised it at line 259 in yellow highlighting.

10. Line no. 261 write programs instead of program

Response: Thank you for your revision. We have revised it at line 272 in yellow highlighting.

11. Line no. 246 write to instead of of

Response: Thank you for your revision. Maybe it is the line of 264? The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references. "The induction of callus" is commonly used.

12. Line no. 265 write induction instead of inductivity

Response: Thank you for your revision. We have revised it at line 278 in yellow highlighting.

13. Line no. 285 write on instead of from

Response: Thank you for your revision. We have revised it at line 294 in yellow highlighting.

14. Line no. 294 write from instead of for

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references, we have revised it at line 304 in yellow highlighting.

15. Line no. 304 write to instead of of

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder at line 339 in yellow highlighting.

16. Line no. 305 write to tend instead of tend

Response: Thank you for your revision. We have revised it at line 340 in yellow highlighting.

17. Line no. 306 write below ground instead of below the ground

Response: Thank you for your revision. We have revised it at line 341 in yellow highlighting.

18. Line no. 311 write induction instead of inductivity

Response: Thank you for your revision. We have revised it at line 345 in yellow highlighting.

19. Line no. 319 write plants instead of plant

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references. "Plant growth" is commonly used.

20. Line no. 323 write subculture instead of subcultured

Response: Thank you for your suggestion. The manuscript was carefully revised by American editor Anita K. Snyder. We have revised it at line 358 in yellow highlighting.

21. Line no. 326 write subculture instead of subculturing

Response: Thank you for your suggestion. The manuscript was carefully revised

by American editor Anita K. Snyder. We have revised it at line 361 in yellow highlighting.

22. Line no. 334 write from instead of of

Response: Thank you for your suggestion. The manuscript was carefully revised by American editor Anita K. Snyder. We have revised it at line 369-370 in yellow highlighting.

23. Line no. 337 write to instead of from

Response: Thank you for your revision. We have revised it at line 371 in yellow highlighting.

24. Line no. 348 write to instead of for

Response: Thank you for your suggestion. The manuscript was carefully revised by American editor Anita K. Snyder. We have revised it at line 380 in yellow highlighting.

25. Line no. 349 write of instead of in

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder. We have revised it at line 380-381 in yellow highlighting.

26. Line no. 354 write from azan instead of formazan

Response: Thank you for your revision. The manuscript was carefully revised by American editor Anita K. Snyder, and we have referred to many related references, Formazan is a blue-violet crystal chemical reagent, which is used to detect the cellular activity. We have revised it at line 386 in yellow highlighting.

27. Line no. 355 write from instead of in

Response: Thank you for your revision. We have revised it at line 387 in yellow highlighting.

28. Line no. 363 write results instead of result

Response: Thank you for your revision. We have revised it at line 402 in yellow highlighting.

29. Line no. 369 write in instead of during

Response: Thank you for your revision. We have revised it at line 408 in yellow highlighting.

30. Line no. 373 write cultures instead of culture

Response: Thank you for your revision. We have revised it at line 410 in yellow highlighting.

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Title: Comparison of the Metabolic Behaviors of Six Systemic Insecticides in a Newly Established Cell Suspension Culture Derived from Tea (Camellia sinensis L.) Leaves

Author: Weiting Jiao, Yizheng Hu, Guoqin Ge, et al

Publication: Journal of Agricultural and Food Chemistry

Publisher: American Chemical Society

Date: Aug 1, 2018

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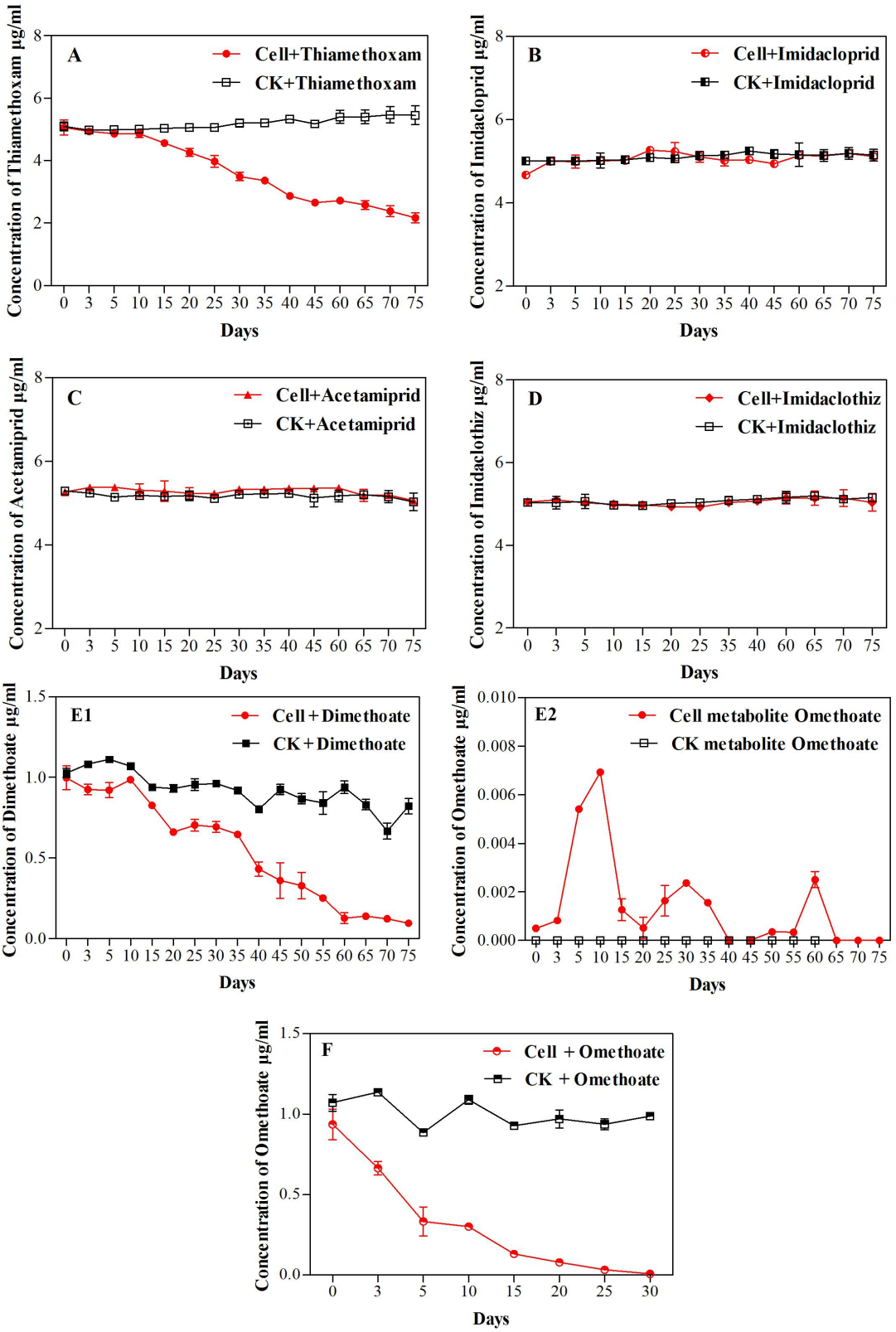

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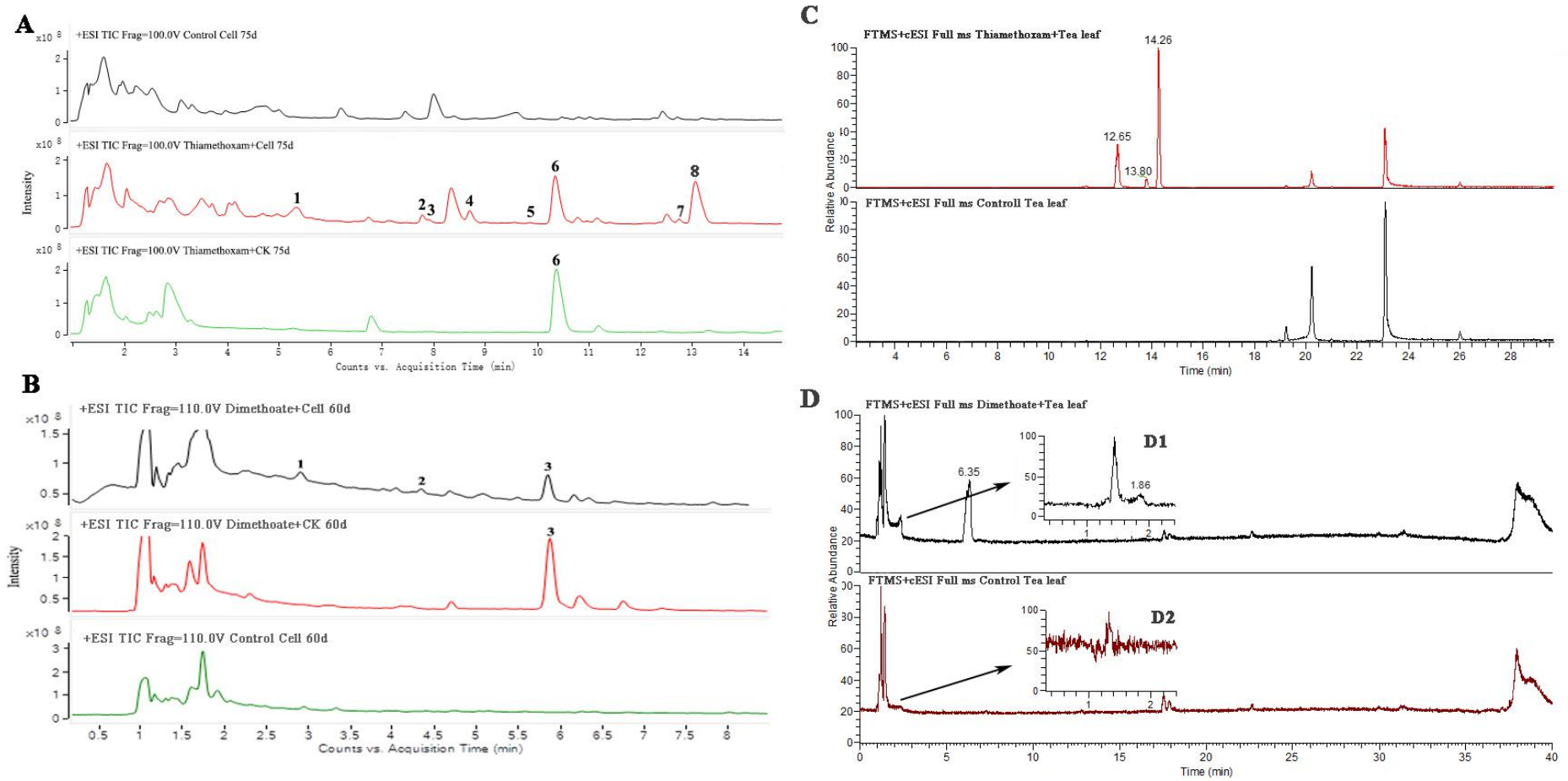

Supplemental Figure 3

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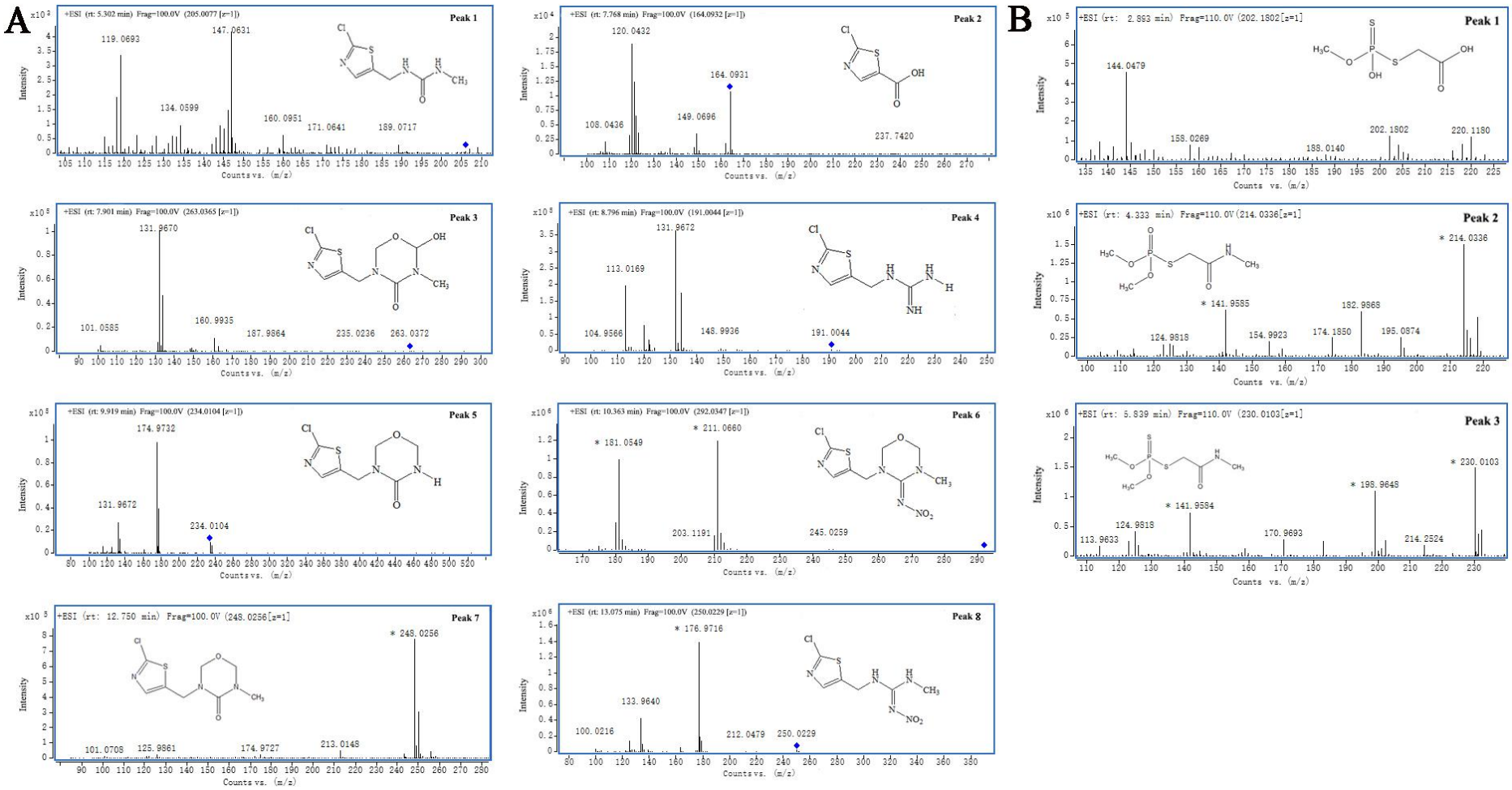

Figure S3. The secondary mass spectrometry using UPLC-QTOF of peaks derived from cultures treated with (A) Thiamethoxam and (B) Dimethoate. *This figure has been modified from [1].*

[1] Weiting Jiao, Yizheng Hu, Guoqin Ge, Jianchao Li, Yu Xiao, Huimei Cai, Lili He, Rimao Hua, Jun Sun, Ruyan Hou. " Comparison of the Metabolic Behaviors of Six Systemic Insecticides in a Newly Established Cell Suspension Culture Derived from Tea (L.) Leaves ", *Journal of Agricultural and Food Chemistry*, 2018, 66, 8593-8601.

Figure 4.