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Exploration of Tritrophic Interaction for Enhancing Conservation Biological Control of Insect Pest, the Role of Analytical Chemistry

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ABSTRACT

Tritrophic interaction among plants, herbivores and natural enemies of the herbivores is mediated by volatiles. Tritrophic interaction model using rice plant, brown planthopper (BPH), and egg parasitoid of BPH were used to study the volatiles involved in the interaction. Extraction of chemicals in rice plants, determination of the extracted volatiles using GC-MS, and bioassays of the volatiles to analyse the orientation behavior of the egg parasitoid were methods used to study the involvement of the volatiles in the interaction. The knowledge of chemistry involved in such complicated natural interaction is beneficial for enhancing conservation biological control (CBC) of BPH using natural population of its egg parasitoid. Implementation of the positive volatile compounds attract the egg parasitoid as a component of CBC, materials involved that adsorb and release the volatiles efficiently. A material made of integrated bio-silica-cellulose was proven to be an effective adsorbent. Analytical chemistry was important in supporting these findings. Furthermore, in order to increase the efficiency of the volatiles that are positively involved in the tritrophic interaction, developing an attractant formula using synthetic-similar compounds and bio-silica-cellulose adsorbent is valuable in enhancing CBC.

Keywords: Biosilica-cellulose adsorbent, chemical cues, tritrophic interaction

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INTRODUCTION

One important phenomenon in nature is tritrophic interaction, in which some volatile compounds are involved. It is the effect of plants on attack of natural enemies like plant parasites. Plant chemistry, morphology and resources will affect the behaviour and performance of natural enemies of the plant parasites (Agrawal, 2000). More modern research in overcoming agricultural problems would take ecological approach rather than using poisonous chemicals to stop the presence of certain insect pests. Some studies of tritrophic interactions have revealed the mechanism of plant defense as well as the consequences in food chain and ecosystem.

When related to plant defense, a model using rice plant, brown planthopper (BPH), and egg parasitoid of BPH (*Anagrus nilaparvatae*) were used to study the volatiles involved in the interaction (Wonorahardjo, Nurindah, Sunarto, Sujak, & Zakia, 2015). Tritrophic interaction is a usual defense mechanism describing ecological impacts of each component which are the plant, herbivore, and parasitoid of the herbivore. While the BPH attack the plants, the wound would induce the plants to produce chemicals that are emitted into the air. This is the sign of host locator for the parasitoids (Agrawal, 2000). The defense mechanism of paddy (*Oryza sativa* L.) is the natural way to make tritrophic interaction work at a bigger scale. In this case the plant was chosen as an example. Biochemistry of the process is really complex but in the end, some new volatiles would be released together with old chemicals to give special scents for the sign of host location. The activation of some genes involved in encoding vegetative storage proteins and repressing genes encoding proteins involved in photosynthetic process is induced by compounds such as jasmonates, as derivations of long chained carboxylic acid (Bi, Zeng, Su, Min, & Luo, 2007) and the presence of these compounds can be found in any plant. The chemical compounds involved in such interactions are mainly called biological volatile organic compounds (BVOCs) and are defined as any organic compounds with vapour pressures high enough under normal conditions to be vaporised into the atmosphere and act as the chemical cues in opportunity or danger (Yuan, Himanen, Holopainen, Chen, & Stewart, 2009).

In tropical lands, there are more complex patterns of tritrophic interactions (Dyer, 2005). The soft climate changing compared to four seasonal places enable the ecosystem with more diverse components to live for multitrophical interactions. The ecosystems in altitudinal gradients show extreme differences in diversity, productivity as well as trophic abundance compared to those from the declined altitude. The ambient temperature and humidity (Dyer, 2005) plays a role for the optimum conditions of more complex possibilities.

It seems that analytical chemistry works in smaller scale of object, and is far too small compared to environmental scale, such as the tritrophic interaction. The task of analytical chemistry is to answer the questions of what and how much in chemistry scale, while environmental scale addresses questions of why and how to describe its phenomena as well as some steps to ‘imitate’ such mechanism.

This study works out the chemical cues in the tritrophic interaction of analytical chemistry. Extraction method provides many possibilities to take the real chemicals under investigation by choosing solvents, and providing the best conditions for the extraction. More apparatus and analyses as well as methods can be set up in modern phytochemical methods, which aim for analysis of secondary metabolites and their roles in wider scopes. After extraction, the analysis for compound separation and identification using GC/MS was done. Some critical analysis must be done to determine the real chemical structures of the volatiles obtained. However, bioassay works with the orientation of parasitoid towards the volatiles give clear indications of preferable chemicals. In the modern investigations, bioassay has become more and more relevant and practical in deciding what chemicals are really involved.

When the description about chemical compounds presence was clear, an attempt for a similar system was also made. The extract of infested paddy containing volatiles were dropped into silica cellulose surface, the volatiles would escape into the air, leaving the surface depending on many physical aspects. This is a trial to make a similar system of wounded paddy stem surface from which the volatiles are released. The creation was based on information from analytical chemistry, which works in chemistry, and is brought into biological spheres.

However, the tritrophic interaction very much depends on spatial and temporal parameters (Raghava, Ravikumar, Hegde, & Kush, 2010). The physical properties of the volatiles as well as the releaser matrix play a significant role. When the place and time refer to humidity, temperature, air pressure, and presence of other chemicals associated with climate changes, then the mechanism of tritrophic interaction alters. Besides that, the intrinsic properties of plants as well as genetic variability amongst cultivars are also important to differentiate the processes. This indicates that complex biochemistry can possibly occur in each condition. With this complex chance and possibility, there are many factors also affecting in changing the release of volatiles. Some scientists have reported an analysis on the role of chemical diversity on defense mechanism, in this case the effect of pollution of ozone layer in changing the metabolism (Iriti & Faoro, 2009).

This proposed system of investigation was implemented under environmental considerations. When analytical chemistry can root the phenomenon from molecular level, then changes from molecular level can occur too. There are the steps of analytical chemistry, starting from sampling and pretreatment to data analysis. The result should be the new set of data for the next treatment. There was also some division of analysis across different levels of objects. The investigations started from chemistry investigation of infested paddy stems. The brown planthopper put the eggs inside the stem by making wounds in the cortex of the paddy stem. The wounds would induce certain biochemistry inside the paddy body and generate some chemicals, some are volatiles and readily spread all over the air. This is chemical cues for the parasitoid. In this case the parasitoid would scent the odor's source. The chemicals are extracted and investigated, and the chemical cues involved in the interaction can be known. To make a similar system, the researchers tried to create a similar situation using adsorbent, and tested them by olfactometer and the parasitoid in bioassay (Wonorahardjo et al., 2015). This is in fact a multidisciplinary approach in overcoming agricultural problems of pests and pesticides. This is also better from the environmentalist point of view by using biological pest controls, in the frame of green chemistry.

In the future, chemistry researches would deal more with biochemistry, to reveal the way some chemicals modulate the genetic expressions. Moreover, the genetic manipulation can also be done to make agriculture beneficial as well as for the environment. On the other hand, analytical chemistry works in different directions. The making of biocontrol pesticides which are environmentally friendly is to fulfill the need for short term necessity. In this case, the role of separation chemistry is also important as the underlying principles of many designs for the bio-pesticides and their carrier materials.

MATERIALS AND METHODS

Analytical chemistry methods can be divided into a few different steps, which were: 1) the sampling and pretreatment of infested paddy, 2) extraction process as well as pre-concentration, 3) separation and analysis by GC/MS, 4) biosilica-nanocellulose adsorbent making, 5) bioassay using parasitoid. All these stages were carried out in chemistry laboratories, at Universitas Negeri Malang, and entomology laboratory, Indonesian Sweetener and Fiber Crops Research Institute (Wonorahardjo, 2013).

The paddy was infested by brown plant hopper and grown under ambient circumstances. Two-month-old plants were chosen for the experiments. The stems of about 50 paddy plants were cut into 5 mm long pieces and immersed in solvent. The extraction was done using soxhlet apparatus and methanol as universal solvent was chosen to take the chemicals out of plant tissues. This polar solvent would be able to dissolve polar and semipolar compounds. The extractions were done in two rows: the healthy paddy as well as infested paddy. All the processes were completed after six circulations. The extracts then underwent pre-concentration using rotary evaporator. The final result was concentrated extract of healthy and infested paddy from the stem part of the plants.

Gas chromatography followed by GC-MS were done for both extracts to know the types of chemicals present in healthy or infested plants. The GC/MS runs were done using Shimadzu instruments (from Shimadzu Singapore). The carrier gas was Helium, the total speed was 20 mL/minute and column speed was 0.5 mL/minute. The initial temperature was 60°C for five minutes, then progressively increased to 300°C before settling it at around 40 minutes. Temperature of ion source was 250°C, interface temperature 305°C with cut time started at minute-3.75. Chromatograms obtained were called as per Library Wiley 8.

An important part of this research is the adsorbent/releaser making. In this study, the adsorbent was made of silica from rice husk ash via sol-gel processing and the method is already used in several occasions for separation purposes (Wonorahardjo et al., 2015). The silica was extracted from the rice husk ash by dissolving the ash into strong base before back gelation by strong acid according to Kalapathy, Proctor and Shultz (2000) with some modifications. In the gelation process, nanocellulose was incorporated into silica porous material. Nano cellulose itself was made by acid hydrolysis of *nata de coco* cellulose fiber for a certain period of time at warm temperature. These methods were registered with Directorate General of Intellectual Properties, Ministry of Law and Human Rights of Indonesia, as a simple patent at the end of 2015 (registration number P00201508272). The cellulose was incorporated into the silica network during gelling, together with sulfuric acid. In this part, nanocellulose was settled on surface of the silica granules.

The physical and chemical characterisation were done using scanning electron microscope (SEM) (PAN Analytical, Singapore) using magnification of up to 10000 times. Besides pictures, porosity and surface area was also done using BET method. Brunauer – Emmett – Teller theory is used to characterise the surface of materials which adsorbs physically gaseous inert molecules (Zhuravlev, 2000). Chemical characterisations were done using water and ash content as well as density and iodine number. Iodine number indicates chemical adsorption of some active molecules with the surface.

Bioassay is a useful method nowadays which includes biological parameters in big amounts. In this experiment, the assay was done using Y-tube olfactometer, in accordance with Lou, Ma and Cheng (2005) with modifications. The olfactometer contains Y-tube, cylinder glass for volatiles' source keeper, aerator, while glass tube contains active carbon as air filter, and cylinder glass with humidifier for keeping filtered air, flow meter light source (Figure 1). Bioassays were done at 8-11 am at 25 ± 2°C.

Figure 1. Olfactometer used in the bioassay step

Validation of the olfactometry was done by testing the parasitoids response to the natural volatiles of the infested plants. The uninfested and infested plants were placed on each odour source. The parasitoids were introduced singly into the Y-tube arm and the orientation of the parasitoid was observed. Parasitoids orientation toward the arm connected to the infected plant volatiles were categorised as having positive response (R+) and those to the uninfested plant were categorised as having negative response (R-). Those which did not show orientation to any arm were categorised as no response (NR). Bioassay of the adsorbent and extracted volatiles on the parasitoid orientation behaviour was done too to validate similar volatiles release from the plant tissue.

Field tests using the adsorbent / releaser and raw extract of infested paddy were done as well. The materials were put into plastic vials and set in wooden stakes and put in rice fields in certain distance. The wooden stake with glued wings to trap the parasitoid would give off the volatiles to attract parasitoid. The number of insects trapped in the glued wings were counted.

RESULTS AND DISCUSSION

The extraction process was the first analytical chemistry done for this project. The raw extract contained more or less the same chemicals as they presented in the plant tissues, before and after the infestation (Figure 2). Changes during process due to elevated temperature, excessive use of solvents in longer immersed time should be taken into account. However, the volatiles which consisted of longer chained hydrocarbon or other groups were relatively stable.

Figure 2. The extraction process and dxtracts obtained from infested and uninfested paddy plants

The different colours of the extracts indicated different contents. Some of them were pigment molecules which were damaged during the process of extraction. The soxhlet extraction is a perfect method in chemistry, however some unstable compounds would be different after extractions. The brown colour of the extract showed degradation compounds together, including the green chlorophyll pigments.

To investigate chemical presence in the extracts, the authors employed gas chromatography followed by mass spectrometry. The compounds were pushed through a semipolar column as the separation process took place. Each compound was carried by Helium carrier gas in certain retention time and would reach the end of the column within different retention time. Soon, after the compounds left the end of separation column, they were directed to the ionisation chamber of the mass spectrometer. Here, fragmentation occurred and the ionic fragments were separated by quadrupolar mass filter to be identified in the detector. From the pattern of fragmentation, the original compound can be matched to the database in Wiley 8.

The raw extracts were both rich of chemicals (Table 1), some were typical volatiles of paddys in other reports in this area (Lou et al., 2005). The similarity may be due to the paddy's types and species, but the difference may be due to different methods, ambient air, altitudes, climate as well as age of the plants (Nurindah, Wonorahardjo, Sunarto, & Sujak, 2017).

Table 1
Complete profiles of chemical compounds composition in infested and uninfested paddy stems (According to Wiley 8 Library)

No	Name of compounds	Uninfested	Infested
		% area	% area
1	3-eicosyne	6,945958	3,991168
2	17-(1,5-dimethyl-hexyl)-10,13-dimethyl-1,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-cyclopenta[a] phenanthren-4-one	6,465718	-
3	Stigmasta-5,22-dien-3-ol	5,447146	-
4	Stigmasta-5,24(28)-dien-3-ol, (3.beta.,24e)-	5,447146	-
5	Eicosanoic acid, methyl ester	5,239284	3,424039
6	9,12,15-octadecatrienoic acid, methyl ester, (z,z,z)-	4,152383	2,926125
7	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [r-[r*,r*-(e)]]-	3,679248	3,290053
8	Hexadecanoic acid, 2,3-dihydroxypropyl ester	3,673684	-
9	9,12-octadecadienoic acid, methyl ester, (e,e)-	3,608907	2,352646
10	Eicosane, 7-hexyl-	3,544913	-
11	Tricosane	3,425545	2,708808
12	Pentatriacontane	3,425545	-
13	Tricosane	3,37482	-
14	Pentacosane	3,37482	4,224462
15	Methyl 5-oxo-2-pyrrolidinecarboxylate #	3,370902	-
16	Hexadecanoic acid	3,24187	-
17	Hexatriacontane	2,989082	4,935695
18	3-hydroxypregn-5-en-20-one	2,98135	-
19	9,12-octadecadienoic acid (z,z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester	2,948544	-
20	3-eicosyne	2,573149	1,525004
21	Octadecane	2,498707	-
22	Hentriacontane	2,354708	-
23	Octacosane	1,850176	4,877568
24	Pentatriacontane	1,665456	-
25	1-isopropoxy-3,3,3-trimethyl-1-[(trimethylsilyl)oxy]disiloxanyl tris(trimethylsilyl) orthosilicate #	1,503278	-
26	3-furylmethanol	1,469662	-
27	Docosane	1,275486	1,662665
28	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	0,914431	-
29	1-decene, 8-methyl-	0,64665	-
30	Benzaldehyde, 4-methyl-	0,613582	-

Table 1 *(continue)*

No	Name of compounds	Uninfested	Infested
		% area	% area
31	(2e)-3,7,11,15-tetramethyl-2-hexadecene #	0,603291	-
32	Phenol, 2,6-dimethoxy-	0,229411	-
33	Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy-	0,226538	-
34	Heptadecane, 3-methyl-	-	4,877568
35	Dotriacontane	-	4,377178
36	Celidoniol, deoxy-	-	4,020032
37	Tetracosane, 11-decyl-	-	3,497129
38	Tetratetracontane	-	3,416504
39	Nonacosanol	-	3,027218
40	9,12,15-octadecatrienoic acid, methyl ester, (z,z,z)-	-	2,926125
41	Heptadecane	-	2,708808
42	9,12-octadecadienoic acid, methyl ester, (e,e)-	-	2,352646
43	Ergost-5-en-3-ol	-	2,271206
44	Ethanol, 2-(dimethylamino)-	-	1,570983
45	2-methoxy-4-vinylphenol	-	0,809865

It is also interesting to see that some chemicals (in red) are present in both healthy and infested plants. In this scheme of thinking, there are natural chemicals which play a role within the tritrophic interactions as equilibrium makers. The blend composition cannot be separated from each other in giving the power as bioattractant, and the biochemistry can go though it in deeper analysis (Jang et al., 2014; Xu et al., 2003). There must be several interesting topics as a sequence of these analytical steps.

In imitating the natural wound-induced volatiles release, hard cellulose materials having the chemicals of the surface were considered. From some investigations the cellulose surface combined with silica can be proven as the slow releaser. The adsorbent made for the bio-attractant gave white porous powder with silica and cellulose content bound together physically (Figure 3). This cellulose on surface shifted the adsorptivity of the surfaces (Wonorahardjo, Wijaya, & Suharti, 2016). This also changed the separation properties of the original adsorbents. By the presence of cellulose, the volatiles were released step by step depending on the temperature, air pressure, as well as surface interactions. The chemical reactions can be written as below:

$$SiO_2(s) + 2\ NaOH\ (aq) \rightarrow Na_2SiO_3(aq) + H_2O\ (l)$$

$$Na_2SiO_3(aq) + H_2SO_4(aq) \rightarrow SiO_2(s) + Na_2SO_4(aq) + H_2O\ (l)$$

Figure 3. Adsorbent making for volatile releaser

Scanning electron microscopy pictures of the material can be seen in Figure 4. The texture of the surface can be seen clearly, that is, the porosity of the matrix plays a role in broadening the surface area. Surface interactions in adsorbing and releasing volatile compounds would regulate the amount of volatiles in the air before regulating the tritrophic interactions.

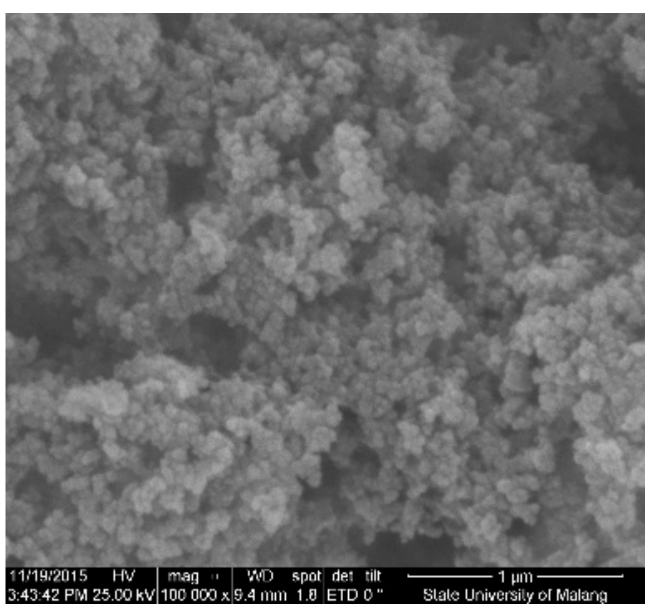

Figure 4. SEM picture of silica-cellulose surface

Adsorbent material made of silica and cellulose can bind the organic content of the extracts in a proper way. Having a good surface area (13.514 m^2/g), the interaction between molecules with the surface can be considered (Table 2). Even though the BET result indicated physical interaction dominant on the surface, the high iodine adsorption numbers also gave an indication of more chemical interaction possible on two types of homogeneous surface (nonpolar and semipolar from cellulose and silica). This is how the act as adsorbent as well as releaser of both the polar and nonpolar compounds is possible (Table 1). Higher water content was the moisturiser of the surface and the system was almost similar to the original wounded-plant stems.

Table 2
BET characterisation, density, water content, ash content, and iodine adsorption number

Characterisation	
Surface area	13.514 m^2/g
Density	3,657 g/mL
Water content	15,400%
Ash content	22,85%
Iodine adsorption number	4,822%

The analytical concepts of equilibrium on surface can be the underlying principles in surface interactions. In this case the solid-gas equilibrium on the interface occurred as far as the surface was covered homogenously by the blended compounds. Moreover, the porous structure they have suggested the in tortuosity played a role in surface interaction. The partly filled porous medium can undergo fast diffusion as studied thoroughly using Nuclear Magnetic Resonance decades before (Ardelean, Mattea, Farrher, Wonorahardjo, & Kimmich, 2003; Mattea, Kimmich, Ardelean, Wonorahardjo, & Farrher, 2004). Strange kinetics on such surface was already studied as the consequences of porous system (Kimmich, 2002). However, in application, discussion about chemistry scale physics was not considered widely. Attention about separation power of some surfaces was given in greater deal.

The biological assessment or bioassay provided useful information for the feasibility of analytical chemistry to get involved in biological research. The olfactometer served as the nature of paddy in field. The positive response towards volatiles from infested paddy was clear. For six experiments, the number of parasitoid which gave positive response was high compared to the negative ones. Only a few numbers showed no response. Since the mixture of compounds was used, blended components cannot be treated separately. The parasitoids involved gave indication for biological activities of the chemicals. This involvement was done since most of the biological systems work with more than one single compound as a blended mixture together. In this case, the knowledge of quantitative effect of each compound is lacking.

Field test using adsorbent/releaser materials in wooden stakes in rice field offered interesting results too (Figure 5). There were also other insects trapped in the glued wings besides the intended parasitoid. The number of parasitoid trapped within five days in a row can be seen in Table 3. In windy situations, more insects can be found. However, more descent experiments must be done to see the results in bioassay for real conditions.

(a) (b)

Figure 5. (a) Plastic vials containing adsorbent/released volatiles; (b) Field test using the source of volatiles

Table 3
Parasitoid entrapment in field test

Day	PSe	JPSe A	JPSa A
I	2	9	10
	0	4	8
II	4	5	7
	2	1	6
III	0	8	5
	0	0	1
IV	3	1	1
	5	0	1
V	1	0	2
	2	2	3

PSe : extract without adsorbent
JPSe A : extract adsorbed in silica cellulose
JPSa A : extract adsorbed in silica cellulose (duplo)

Within five days, the number of molecules given off from the surface of silica-cellulose would be decreased. In warmer temperature, the age of this bioattractant would be shorter. There must be more treatment in the surface to be applied so that the sequence of evaporating molecules can be calculated. In this case, the age of the attractant in the field, as far as the rain does not come, can be determined. Moreover, the expiring date of the formula must be given as well.

There is a lot to analyse in every step of analytical chemistry. The sensitivity, selectivity, and effectiveness of the bio-attractant must be followed too. When it goes deeper into plant biochemistry, more new insights can become key knowledge for the development of friendlier biological controls.

CONCLUSION

There are a lot of lessons learned from the natural behaviour of living things on earth. The mechanism of defense is one of the natural phenomenon and can be followed at least by analytical chemistry. The steps to analyse and imitate tritrophic interaction were done using paddy-brown planthopper-egg parasitoid system, from extraction and analysis followed by making adsorbent/releaser of volatiles, bioassay as well as field study. More analysis on bio-attractant making should be done in future, but the complex biochemical changes in between the steps are the objects of much follow-up research. Similarly, the study of surface interaction and material making can be crucial for agricultural purposes. The type of industrial bio-attractant can be considered and for that, analytical parameters should be set and optimised before they are applied.

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