Effectiveness and safety of botanical pesticides applied in black pepper (*Piper nigrum*) plantations.

W i r a t n o

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~ Dedicated to my beloved wife Mawaddatul Jamilah and our daughters Syifa, Putri, and Dhelia ~

Cover description:

- Front cover: The top picture describes an old woman is drying pepper berries in the sunlight using traditional equipment. The bottom pictures shows steps performed in the field experiment; a volunteer sprays insecticides to canopies of pepper plants (left picture), observation of the fallen dead insects after pesticide application (middle picture), and healthy pepper berries (right picture).
- Back cover: Three insect pests used as model species during laboratory experiments. At the top is *Lophobaris piperis*, in the middle *ferrisia virgata*, and at the bottom is *Aphis gosypii.*

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Preface

It was beyond my thoughts that unexpectedly I had the opportunity to do a PhD study. An extra bonus for me was that the study had to be completed in one of the most acknowledged universities in Europe, Wageningen University, the Netherlands. I am therefore grateful to the people who made my dream become reality.

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Finally, all the praises and thanks to Allah, the Lord of the Alameen, the most Beneficent, the Most Merciful.

Wiratno

Chapter

1

General Introduction

The research described in this thesis concerns the evaluation of the effectiveness and safety of plant extracts as potential botanical pesticides. Seventeen plant extracts and formulations of selected extracts were tested for pesticidal activity against representative pest species. The research included laboratory, green house and field experiments. The laboratory experiments were partially conducted in the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI) and at the laboratory of Nematology of Wageningen University, the Netherlands. The green house experiment was conducted at the IMACRI. The field experiments were performed in the centre of Indonesian pepper plantations i.e. on Bangka Island and in the Lampung province. The research was performed within the framework of the Integrated Pest Management for Smallholder and Estate Crop Project (IPMSECP). The project focused on protection of black pepper (*Piper nigrum* L) against pests.

1.1. General Introduction

Black pepper (*Piper nigrum* L) is an important commodity of Indonesia, which has been cultivated within the country since the 6th century. The widest growing areas are found in the provinces of Lampung, West Kalimantan and Bangka-Belitung. Pepper has been exploited both as a national export product and as raw materials for some industries, producing foods, medicines, and cosmetics. The plant plays an important role in local economies since 95% of the plantations are cultivated by smallholder farmers [1)]. Indonesia is one of the most important pepper exporting countries in the world contributing 90% of the whole pepper market together with India, Brazil, and Malaysia [2)]. Because of this important economic value, proper pepper plant production is highly valued. One of the central factors is the control of pests in the pepper plantation.

1.1.1. Key pests of black pepper and control strategies

The key pest species of black pepper are either attacking the plant underground or above ground. One of the most important underground pests is *Meloidogyne incognita* [3)]. This species is a member of a major group of plant-parasitic nematodes affecting both the quantity and quality of crop production [4)]. On Bangka Island, *M. incognita* is considered to be a major problem in pepper plantations. In 2003, 4.900 ha of the total of 52.468 ha of pepper plantations was severely infected by this organism [5)]. Although, there is no qualitative information about the exact loss of pepper production due to nematode infection, it is clear from visible inspection that severely attacked plants have a reduced vitality, hardly produce berries, and finally die [6].

The most important insect pests attacking the upper part of pepper plants are the stem-borer, *Lophobaris piperis*, the tinged bug, *Dasynus piperis* and the bug, *Diconocoris hewetti*. The stem borer is a small pepper weevil of which the larvae bore holes in the stems of the pepper plants. Especially when the climbing stem of the plant is damaged further growth of young plants will be hampered [3]. The tinged bug is a large pepper berry bug that not only causes losses because of falling down of part of the fruits but especially causes serious damage when the damaged berries get infected with *Colletotrichum* sp, fungi causing fruit-bunch-rot. The bug feeds exclusively on pepper species by sucking the inflorescence and the very young fruit bunches. The amount of damage is dependent on the duration of the flowering period. If the flowering only occurs during a discrete period, the damage is limited. However, if cultural measures extend flowering throughout the year, the number of bugs increase greatly [7].

In order to minimize plant damage caused by pests, at present farmers mainly depend on the use of synthetic pesticides [8]. Nowadays, about 20 pesticides are recommended for controlling pests of black pepper [9]. However, the use of synthetic pesticides in most of the developing countries, including Indonesia, is frequently associated with inappropriate training and unsafe application of the pesticides. The flawed use and disposal of pesticides poses not only a serious health risk to local workers and the people living near the treated areas, but also threatens non-target species, including potential natural enemies of the pests [10]. Therefore, it has become an important issue to find relatively easy alternative control strategies, which are as effective when compared to the synthetic pesticides, but safer to the farmers, consumers, and the environment and available at low price [11].

One of the possible alternatives would be the use of pesticides of plant origin, also known as botanical pesticides. Botanical pesticides have been used by man since ancient times, especially in cultures with a strong herbal tradition [12]. They have been reported to be effective against i.e. nematodes [13, 14], beetles [15-18], mites [19], ticks [20] and fungi causing plant diseases [21-23]. Parts of the plants which are used for the pesticides are roots or rhizomes (i.e. of derris) [24], vetiver [25] and sweet flag [16], flowers or buds (i.e. pyrethrum) [26] and clove [27]), seeds (i.e. neem) [28], castor bean [29] and yam bean [24]), and leafs (i.e. patchouli) [30], betelvine [31] and tobacco [32]). Although the mechanisms of action of the botanical pesticides may differ greatly and are often not yet well understood, they have as advantage that they combine a wide range of toxic potencies hence reducing the chance of pests to develop resistances [33]. In addition to that, residues are hardly expected on the products or in the environment since botanical pesticides are generally considered to be non-persistent under field conditions as they are readily degraded by light, oxygen and micro-organisms into less toxic products [34].

However, since botanical pesticides are non-persistent, application of the pesticides has to be repeated more often compared to that of the synthetic pesticides.

Indonesia seems to be in a good position to develop and utilize botanical pesticides since the country has a rich biodiversity of plant species [35)] some of which have already been used as a pesticide [36)]. Nowadays, the increased consumer request in developed countries for organic products which are free from synthetic pesticide residues stimulates the interest in the use of botanical pesticides in agricultural production by exporting tropical countries [37)].

1.1.2. Objectives of the research

The overall objective of the research described in the present thesis was to study the effectiveness and potential of pesticides of plant origin for application in black pepper plantations.

1.1.3. Steps to reach the objective

To reach this objective the project consists of the following steps:

Making an overview of the current methods of application of synthetic pesticides in black pepper plantations and the possible associated risks (chapter 2);

Determination of the toxic potencies of seventeen Indonesian plant species known from ancient times or literature to potentially have nematicidal and pesticidal properties and therefore could be developed into botanical pesticides (chapter 3, 4 and 5);

Development of formulations of the most promising plant extracts as botanical pesticide and testing of both their effectiveness to control above ground and underground pests plus their toxicity to non-target terrestrial and aquatic organisms (chapter 3 and 5).

Evaluation of the potential risk for farmers and consumers arising from applying this botanical pesticide (chapter 6).

Ad 1) To answer the question whether farmers apply synthetic pesticides in pepper plantation wisely or whether the way they are applied pose a risk to local workers, consumers and the environment, a baseline study was performed on Bangka Island where pepper is cultivated intensively. This baseline study included questioning 117 local farmers about their habits in pesticide use and determining pesticide residues on the berries as well as those in the urine, the blood, and on the body of 2 volunteers spraying a synthetic pesticide according to routine farmer procedures. In addition to this study, the potential risks of synthetic pesticide exposure to local non target terrestrial and aquatic invertebrates were evaluated via ecotoxicological modelling (Chapter 2).

Ad 2) To assess whether botanical pesticides have comparable potencies as synthetic pesticides to control pests of black pepper, laboratory bioassays were performed. Chapter 3 describes the nematicidal activity of the 17 plant extracts against the root-knot nematode, M. incognita. The 2 plants providing the most promising extracts were subsequently assayed in a green house experiment to evaluate their effectiveness compared to that of a recommended synthetic pesticide in controlling a root-knot nematode when applied as a mulch of raw materials. Chapter 4 describes the contact toxicity, oral toxicity and repellency of 17 plant extracts against the model insect species Tribolium castaneum to find the most potent plant extracts, which would be tested against L. piperis, a key pest of black pepper.

Ad 3) Formulation of three of 10 most potent extracts against *L. piperis* was developed and tested in the laboratory against L. piperis and 3 other pest species of pepper plants i.e. *Ferrisia virgata, Aphid gossypii* and *A. craccivora*. Evaluation of the formulation also included evaluation of its toxicity against mosquito larvae representing aquatic non-target organisms. Finally the effectiveness of the formulation was compared with that of the recommended synthetic pesticide against pests of black pepper in the field and against non-targeted organisms such as insect natural enemies and insect pollinators (Chapter 5).

Ad 4) The safety of five botanical pesticides that, based on the results of the present thesis, were most promising for use as botanical pesticides, was evaluated for human oral exposure via consumption of treated products. Based on literature data from human and animal studies safe levels for daily oral exposure to the various botanical preparations and/or their active ingredients were derived and these outcomes were compared to the estimated maximal daily intake of residues of the botanical pesticides expected to be present on pepper berries treated with these preparations as pesticides (Chapter 6).

Finally, in chapter 7, the implications of the presented work are discussed, with emphasis on the potency, suitability and the safety of the botanical pesticides when used against pests associated with pepper plants.

Chapter

2

A Case Study on Bangka Island, Indonesia on the Habits and Consequences of Pesticide Use in Pepper Plantations

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Abstract

Habits and consequences of pesticide use in black pepper plantations were studied in Indonesia. The first study was conducted by questioning 117 farmers about their habits in pesticide use and determining pesticide residues on both exported pepper berries and berries on the local market on Bangka Island. Meanwhile, the second study was completed by analyzing exposure levels of pesticide in farmers' bodies before and after pesticide application to pepper plantations at Sukamulya, West Java. Risks of pesticide exposure to below ground terrestrial invertebrates and aquatic ecosystems adjacent to the treated fields were evaluated using scenarios and a decision support system. Results showed that 5 respondents (4.3%) were agricultural workers without an own plantation, the others were plantation owners. About 112 respondents (95.7%) used pesticides regularly, while 21 respondents (17.9%) had experienced pesticide poisoning. About 54 respondents (46.2%) tended to apply the same pesticide on all occasion and 104 respondents (88.9%) indicated to always apply a single compound. About 91 respondents (77.8%) were not aware of the possible impact of pesticides on their health and 102 respondents (87.2%) were not aware of the possible effects on the environment. In addition while spraying pesticides 17

respondents (14.5%) were smoking, 81 respondents (69.2%) were wearing daily clothes and 84 respondents (71.8%) were throwing empty bottles into the forest. Exposure study revealed that the residues in the urine and blood increased 6.5-10 and 1.1-1.5 folds, respectively indicating actual and direct exposures. The environmental risk assessment indicated low risks for the terrestrial below ground invertebrates but high potential risks for the aquatic ecosystem. The residues of the major pesticides were below the maximum residue limits. This case study indicated that the farmers and their workers, and probably also the environment were at risk of high exposure to the pesticides applied, but that the risks for the consumers were negligible if present at all.

Key words: black pepper, developing country, Indonesia, Pesticide use

2.1. Introduction

Black pepper (Piper nigrum L.) known as the “King” of spices is economically the most important and worldwide the most widely used spice crop. This leading position among spices results in increasing commercial value in the world trade and it is predicted that the global demand for pepper will increase from 230 000 metric tons in 2010 to about 280 000 metric tons by the year 2020, possibly further increasing to 360 000 metric tons by 2050 [38)].

In Indonesia, pepper was originally introduced by Hindu colonists between 100 BC and AD 600. The most important growing areas for pepper are Lampung producing Lampong black pepper, Bangka producing Munthok white pepper, and West Kalimantan producing black and white pepper. Nowadays, pepper economically is one of the most important commodities in those areas also because, being a labour intensive crop, it provides jobs for the local population. In addition to being a source of national revenues, pepper can be exploited as a source of raw materials for some industrial products, such as food, medicines, and cosmetics [39)].

Because nowadays farmers cultivate pepper intensively, pesticides are applied in high dosages and frequencies, especially to control the most important pests in the plantations, which according to Kalshoven [3)] are tinged bug (*Dasynus piperis* China.), stem borer (*Lophobaris piperis* Marsh.), bug (*Diconocoris hewetti* Dist.) and root-knot nematode, (*Meloidogyne incognita* Kofoid and White.). Department of Agriculture of Indonesia [40)] provides the synthetic pesticides which until now are recommended by the Indonesian government to fight those pests. Most of the pesticides used are insecticides, namely pyrethroids (Pyr) or organophosphates (OP) or carbamates (Carb), and two herbicides i.e. Paraquat and glyphosate.

This intensive use of synthetic pesticides could, however, have serious implications for the health of the farmers and their families, consumers, live stock and the environment. Jeyaratnam [41)] estimated there were 30000 cases of pesticide poisoning annually in

Indonesia, of which approximately 2400 required hospitalization. Currently both the Indonesian government and consumers increasingly demand healthier and environmentally friendlier products. Therefore a study was performed on the current farmer habits and consequences of the use of synthetic pesticides in pepper plantations, especially on Bangka Island.

The aim of this study was **i)** to obtain information on the consequences of the crop protecting practices using synthetic pesticides for local people, on the awareness of people working with pesticide about health and environmental risks and actual poisoning cases, on residual levels of major insecticides on pepper berries, on exposure of farmers to pesticides during application, and **ii)** to perform an estimated environmental risk assessment of some recommended pesticides. It is expected that this information can be used by the local government to improve the quality of life of the farmers and support the necessity to look for alternative methods for crop protection.

2.2. Materials and Methods

2.2.1. Selection of the study areas and respondents

The study was conducted from June until August 2004 on Bangka Island, one of pepper central productions in Indonesia. General information related to the production volume and area of all commodities was collected and compiled from data from districts and provincial offices. Detailed information concerning farmers' behaviour in managing their plantations was gathered through a field survey. The selection of the locations to interview farmers was based on purpose sampling to obtain convenience samples, in which districts, sub-districts, and villages would not be chosen if only a few black pepper plants were grown there. Moreover, farmers only were chosen if they applied pesticides by themselves. If they worked e.g. as tin miners, fisherman, officers or shopkeepers they were excluded as respondents. Out of 5 districts of Bangka Island, 4 districts (80%) were selected and in each of them 2-3 sub-districts were selected (40.5%). From each sub-district 4 villages were selected (34.8%) and from each village 3-5 farmers were interviewed, depending on the number of farmers that was available during the study. In total 117 respondents were questioned. The questionnaires focused on the farmers' understanding of the possible risks for man and environment when using pesticides reflecting the way they worked with those toxic compounds. Detailed questions of the questionnaires were related to major pesticides used by farmers, dosages or concentrations of pesticides being applied, time and method of pesticide application, poisoning cases of workers, and behaviours of local farmers when using pesticides with respect to the use of protective clothing while spraying, the moment of smoking cigarettes related to spraying activities, and the way of disposal of emptied pesticide containers.

2.2.2. Farmer exposure study

This study was conducted on August 18th, 2004 at the Research Installation of the Indonesian Spice and Industrial Crops Research Institute (RIISICRI), Sukamulya. During the study, two officers who also often used to spray pesticides to control pepper pests in the experimental garden of this institute were requested as our volunteers to spray chlorpyrifos onto 100 pepper plants according to common practices of the interviewed farmers. Before commencing the application, the volunteers were informed about the risk and possible effects of the pesticide to their health in accordance with the ethical concerns and rules of the Indonesian Medicinal and Aromatic Crops Research Institute for involving people in working with toxic compounds.

Spraying was carried out in clear weather with 29ºC and 67% humidity. The first farmer who sprayed 3 ml l^{-1} concentration was wearing long sleeves and covered his head with a t-shirt. The other farmer who sprayed 1 ml l^{-1} concentration was wearing a casual t-shirt and covered his head with a cap. Application was completed using 15 l Solo knapsack sprayers. Application began at 9 am and lasted for 2 hours, with a short break for 15 minutes at 10 am. About 50 ml of urine and 5 cc of blood were sampled before (8.00 am) and after application (2.30 pm) from the volunteers at the Medika Laboratory, Cibadak, Sukabumi. In order to avoid blood coagulation ± 50mg of ethylene diamine tetra-acetic acid (EDTA) was added. To estimate the pesticide residue on the farmers' clothes, a piece of 20x20 cm^2 tissue paper was attacked on the chest of each volunteer before spraying commenced. All samples were put in glass bottles and cooled until they could be transferred to a freezer at -4ºC in the laboratory. Two days later the residue levels of major pesticides were analyzed using gas chromatography as described above.

Extraction procedures of tissue papers, urine, and blood samples for gas chromatography analysis were almost similar. However, the organic solvent for tissue paper was acetone absolute, while that for urine and blood was a mixture of n-hexane and dichloromethane (60:40). Details of the extraction procedures were as follows; firstly, 50 ml urine or a tissue paper was dissolved in 100ml organic solvent and shaken using a mechanic shaker at 40 rpm for 20 minutes, while 5 cc bloods was dissolved in 10 ml solvent then was shaken in a vortex for about 2 minutes. After that the solvent, containing the extracted pesticide was collected. The remaining sample was then re-extracted twice using the same solvent and procedure. The samples of the three extraction steps were mixed and homogenized. After that the solvent was evaporated using a rotary evaporator until about 1 ml was left. The sample was then purified in a chromatography column filled with 30 g florisil and sodium sulphate anhydrite and wetted with 50 ml solvent. After that the sample was re-evaporated using a rotary evaporator until the volume of the solution was about 1 ml then the solution was transferred into a trial tube. Lastly, the inner part of the evaporating glass was washed step by step using 9 ml solvent to make sure that the pesticide residue was completely dissolved then the solution was poured into the above trial tube. Of the 10 ml of the final product, 2 µl of the solution was used for the GC analysis.

2.2.3. Chemical analyses

The residue levels of the major insecticides used by the farmers were determined on pepper berries obtained from both the local market in Belinyu and two large exporters in Pangkalpinang, the Capital City of Bangka Island. The residues were analyzed in the Indonesian Centre for Agricultural Biotechnology and Genetic Resources Research and Development. The details of the gas chromatographic conditions used were as follows;

A gas chromatograph (Shimadzu 4 CM) equipped with Electron Capture Detector (ECD) and glass capillary column (2m length, 3mm diameter containing chromosorb waw) was used. The injected volume was 2 µl and nitrogen ultra high pure (N2UHP) gas was used as carrier at a flow rate of 40ml min^{-1}. The injector and detector temperatures were 220 and 230^{o}C, respectively. The sensitivity of the GC was set manually to 10^2 MΏ and the pulse was 10(H) kHz. The detection limits for chlorpyrifos, lambda-cyhalothrin, and BPMC (2-sec-butylphenyl methyl carbamate) were 0.0002, 0.0038, and 0.0012µg g-1 of samples, respectively.

2.2.4. Environmental Risk Assessment

The risks of the pesticide use to the environment were assessed using hypothetical scenarios applying the risk assessment model PRIMET (Pesticides Risks in the Tropics to Man, Environment and Trade) [42)]. In order to perform a risk assessment in PRIMET a scenario describing the physical properties of the environmental compartment must be provided. This scenario was combined with usage data and some pesticide properties to calculate a Predicted Environmental Concentration (PEC). This PEC was then compared to a No Effect Concentration (NEC) to calculate the Exposure Toxicity Ratio (ETR). The NEC was calculated from EC50 values based on laboratory toxicity tests performed with standard test species and safety factors to account for between species variability and the extrapolation from 50% effect to no effect. The procedure to calculate the PEC and NEC was in accordance to the EU regulations [43)] and was described in detail in [42)]. An ETR lower than 1 indicates that no serious effects could be expected, one of between 1 and 100 that effects were uncertain while an ETR of higher than 100 indicated that effects were likely to occur. The risks were evaluated for an adjacent aquatic ecosystem and in-crop below ground terrestrial invertebrates.

The aquatic scenario assumed an aquatic waterway of 1 meter wide at the bottom, a slope of 0.5 and 50 cm of water depth. The length from which the ditch received spray drift following the applications as provided in Table 1 was 100 m with a flow velocity of 100 m day^{-1}. The water phase was assumed to contain 1 g L^{-1} of suspended solids with an organic matter content of 50%, while the water temperature was assumed to be 30°C. It was assumed that 10% of the amount applied on the soil surface would reach the water surface by spray drift. All these values seem to be realistic for a tropical scenario, see e.g. [44)].

The soil scenario assumed a soil with a bulk density of 1100 kg m^{-3} and the top 5 cm would contain the invertebrates at risk.

The toxicity values for the aquatic and terrestrial standard test organisms were obtained from the National Institute of Public Health and the Environment, The Netherlands (RIVM) database ([45, 46)], respectively. For the application scenario the usage data as provided in Table 1 were taken to calculate grams of active ingredients applied per hectare.

The PRIMET DSS (Decision Support System) is freely available on http://www.primet.wur.nl and incorporated in a Graphical User Interface.

2.3. Results

Results of the secondary data collections from provincial and district offices showed that pepper was the most common plant grown by farmers on Bangka Island, followed by rubber, coconut, oil palm, cacao and clove, respectively (Table.2). Though pepper was cultivated intensively in most districts of the study areas except in Pangkalpinang, wide areas of pepper plantations within the sub districts and villages were still diverse [47)]. Therefore, sub districts and villages would be selected only if it had the widest areas of pepper plantation.

The baseline study revealed that 23 respondents (19.7%) cultivated less than 500 plants, while 56 respondents (47.9%) had 500 to 1000 plants and 38 respondents (32.5%) even grew more than 1000 black pepper plants. The study also revealed that 112 respondents (85.7%) regularly applied synthetic pesticides to control main insect pests of black pepper i.e. the stem borer (*L. piperis*), the tinged bug (*D. piperis*), and the bug (*D. Hewetti*), and in addition to control weeds. Though carbofuran had been recommended by the ministry of agriculture to control the root-knot nematode, *M. incognita* [48)], only few respondents used this pesticide due to based on their experiences there were no pesticides effective enough to control this pest. The amount of pesticides used by the respondents depended on the price of the pepper berries and the population density of pests or weeds. Generally, most respondents (98.3%) used the cap of pesticides' containers (8 ml) to measure the amount of pesticide to be used. Most of the respondents (91.5%) used 2 or 3 caps of pesticide per 15 litter of water, which was equivalent to 1.1 or 1.6 ml l^{-1}, while the rest used 4 caps (equivalent to 2.1 ml l^{-1}). The recommended concentrations of pesticides varied between 0.1 – 3 ml l^{-1}. In very extreme conditions in which price of the berries or density of the pest population was quite high, respondents would use 4 or even until 5 caps pesticide which was equivalent to 2 – 2.5 ml l^{-1}. Based on this information, some pesticides were applied in higher concentrations than that of the recommended concentrations, especially those belong to pyrethroid group i.e. cypermethrin, beta-cyfluthrin, deltamethrin, lambda-cyhalothrin, and fenpropathrine (Table 2). The richer farmers tended to use more pesticides than the underprivileged farmers who only could apply 1-2 caps per 15 l of water equivalent to 0.5 – 1.1 ml l^{-1}.

Table. 1 Recommended synthetic pesticides to fight the most important pests in black pepper plantations [49], including their active ingredients, purity and dosage

Pesticides	Active ingredients	Dosages/ concentration	Vol. of Appl. (l ha^{-1})	Max dose applied (g ha^{-1})
Ambush 2EC	Permethrin 20g l^{-1}	0.5-l.0 l ha^{-1}	nd	10
Arrivo 30EC	Cypermethrin 30.36g l^{-1}	0.5-1.0 ml l^{-1}	1200-1500	46
Bassa 500EC	BPMC 500 %	Na	na	na
Buldok 25EC	Beta-cyfluthrin 25g l^{-1}	0.5-1.0 l ha^{-1}	nd	25
Decis 2.5EC	Deltamethrin 25g l^{-1}	0.1-0.2 ml l^{-1}	nd	na
Dharmacin 50WP	MIPC 50%	na	na	na
Dharmasan 600EC	Fenthoate 600g l^{-1}	na	na	na
Dursban 20EC	Chlorpyrifos 200g l^{-1}	1.0-2.0 ml l^{-1}	1000	400
Elsan 60EC	Fenthoate 60g l^{-1}	2.0 ml l^{-1}	500-800	96
Lebaycid 500EC	Fenthion 500g l-1	2.0 ml l^{-1}	nd	na
Matador 25EC	Lambda-Cyhalothrin 25gl^{-1}	0.5-1.0 l ha^{-1}	nd	25
Marshal 200EC	Carbosulfan 200g l^{-1}	1.5-3.0 ml l^{-1}	1200-1500	900
Mipcin 50WP	Isoprocarb 50%	1.0-2.0 kg ha^{-1}	nd	1000
Meothrin 50EC	Fenpropathrine 50g l^{-1}	0.5-1.0 ml l^{-1}	nd	na
Orthene 75SP	Acephate 75%	na	na	na
Padan 50SP	Cartap hydrochloride 50%	2.0 kg ha^{-1}	600-800	1000
Pounce 20EC	Permethrin 20.04g l^{-1}	1.0-2.0 ml l^{-1}	nd	na
Sevin 85AS	Carbaryl 85%	2.5 kg ha^{-1}	500-1000	2125
Sumithion 50EC	Fenitrothion 500g l^{-1}	na	na	na
Sumicidin 5EC	Fenvalerate 44.5g l^{-1}	na	na	na
Furadan 3G	Carbofuran 3%	30.0 gr $plant^{-1}$	nd	na
Gramoxone	Paraquat dichloride 276gl^{-1}	2.0-3.0 l ha^{-1}	nd	828
Sunup 480AS	Glyphosate 480g l^{-1}	na	na	na

The pesticides used are insecticides, namely pyrethroids (Pyr) or organophosphates (OP) or carbamates (Carb), nematicide (Carb) and herbicides, namely Paraquat dichloride (bipiridillium) and N-(phosphonomethyl) glyphosate. a Recommended dosages or concentrations found on the packages. na, Pesticide is not available on local markets. nd, data are not available on the packages.

The most popular pesticides were fenthion, lambda-cyhalothrin, and BPMC (2-sec-butylphenyl methylcarbamate), while only one respondent still used tuba root (*Derris elliptica* Benth.) (Figure 1). About 40 respondents (45.8%) tended to apply the same insecticide all the time, while 104 respondents (89%) preferred to apply single compounds. The rest would mix pesticide with fertilizers or other compounds such as fungicides or cajuput oil, the oil which was extracted from *Melaleuca leucadendron* L. was believed able to repel pests in plantations.

Table. 2. Overview per crop of the surface area and production of smallholder plantations in 4 districts of Bangka Island [50].

Commodities	Districts								Total	
	Main Bangka		Central Bangka		South Bangka		West Bangka			
	Width area (ha)	Prod. (tons)	Width area (ha)	Prod. (tons)	Width area (ha)	Prod. (tons)	Width area (ha)	Prod. (tons)	Width area (ha)	Prod. (tons)
Pepper	13,725.00	5,388.58	5,940.08	1,907.36	1,8621.58	12,830.63	10,229.90	3,440.03	48,516.56	23,566.60
Rubber	18,314.35	7,327.50	2,733.00	1,001.00	4,448.40	1,910.35	13,157.00	4,017.00	38,625.75	14,255.85
Coconut	5,956.50	3,064.40	2,497.30	1,234.29	1177.53	468.82	2,416.15	975.30	12,047.48	102.81
Oil palm	815.00	237.65	152.00	79.16	24.00	15.00	407.00	237.00	1,398.00	568.81
Cacao	140.00	43.20	51.00	-	730.00	43.20	25.50	0.10	289.50	86.50
Clove	71.00	0.64	49.00	4.18	33.25	0.78	102.50	0.85	256.21	6.45
Sugar Palm	77.00	24.15	53.00	17.31	38.50	0.83	39.00	14.25	207.50	66.54
Cashew	63.00	22.16	20.00	8.80	100.00	7.24	13.40	1.89	196.40	40.09
Betel Palm	27.27	1.21	75.82	3.83	37.30	1.27	26.31	8.64	166.70	14.95
Candle Nut	10.50	-	46.00	7.03	9.00	6.01	6.00	-	71.50	13.04
Coffee	-	4.02	32.00	0.20	18.00	-	9.00	3.66	59.00	7.88
Gambier	32.25	-	-	-	-	-	7.00	-	39.25	0.00
Patchouli	15.00	0.93	-	-	11.00	-	4.00	1.25	30.00	2.18
Ginger	-	-	-	-	-	-	-	0.15	0.00	0.15
Tee	-	-	-	-	-	-	1.00	-	1.00	0.00

- Data are not available

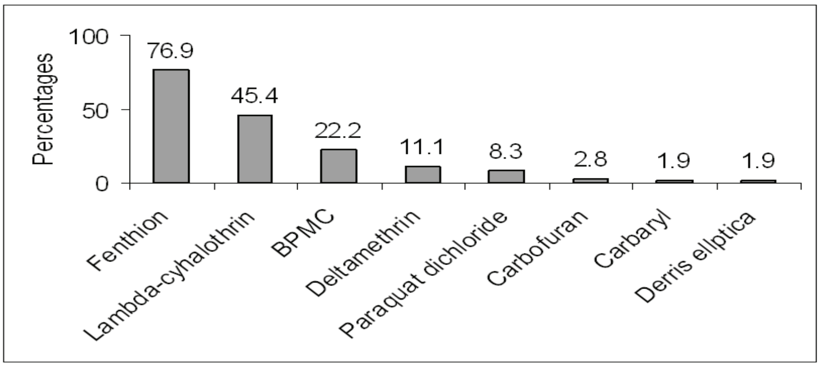

Figure 1. Percentage of farmers using the pesticides specified in black pepper plantation on Bangka Island based on interviews with 117 respondents

Of the respondents 92.3% (108 respondents) preferred to only spray pesticides directly onto their plants while the rest immersed the pesticide into the rooting areas especially to control M. incognita. Application would start around 7.00 am and continue until 12.00 am and would be continued from 1.00 pm until 5.00 pm. However, the period of application of pesticides differed among the respondents. About 69 respondents (58.9%) sprayed both in the morning and in the afternoon, while 46 respondents (39.3%) only sprayed in the morning and 2 respondents (1.7%) only sprayed in the afternoon. There were 113 respondents (96.6%) immediately took a bath and washed their sprayer tanks and clothes in the river after application of the pesticides. However 9 respondents (7.7%) just hung up their clothes and would use them again the following day. They would wash those clothes after the second application or occasionally after finishing spraying all plants.

Unfortunately, 90 respondents (76.9%) did not really realize the possible impact of pesticides on their health. As a result about 21 respondents (17.9%) felt pesticide poisoning though they did not visit physicians for further clinical investigation. Because of the generally limited awareness of possible health risks during application of pesticides, 81 respondents (69.2%) just wore their ordinary clothes without considering any protection against contamination with pesticides (Figure 2a). Only 1 respondent (0.9%) fully protected himself. During spraying pesticides about 18 respondents (15.4%) even smoked a cigarette, and 77 respondents (65.8) smoked during the break before washing or changing clothes (Figure 2b). Most of the respondents would take a bath before having lunch. This habit, however, was due to the hot weather causing strong sweating and not because of awareness of the risk of using pesticides. The awareness of the importance of safe disposal of the empty pesticide containers seems to be very limited as 82 respondents(70.1%) just threw empty pesticide containers away into the forest and 1 respondent (0.8%) even used them for other purposes such as to make lamps (Figure 2c). In addition, of the respondents 102 respondents (87.2%) did not really realize the existence of natural enemies and their important role in controlling pests in their plantations

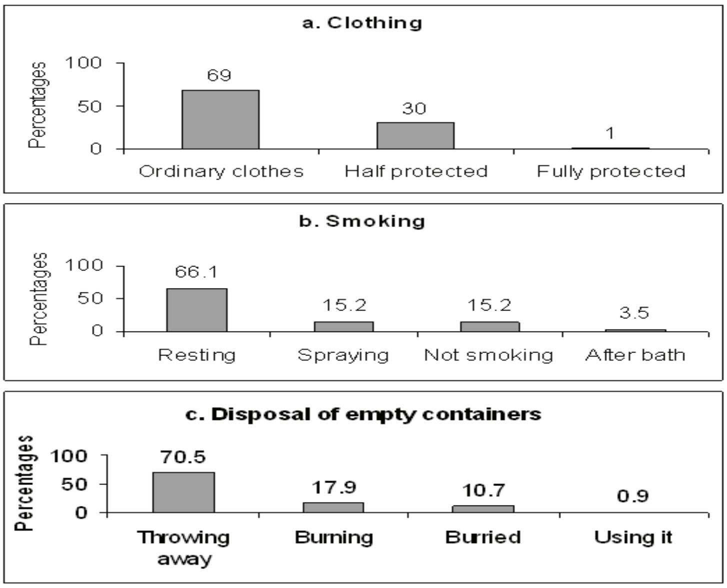

Figure 2. Percentage of local farmers on Bangka Island, Indonesia displaying specific behaviour when using pesticides in black pepper plantations with respect to a) the use of protective clothing while spraying, b) the moment of smoking cigarettes related to spraying activities and c) the way of disposal of empty pesticide containers.

The two volunteers in the exposure study sprayed the pesticide in the common way as the interviewed farmers. They walked around the plant trying to spray the canopy of pepper plants evenly. They used one hand to pump the tank while the other hand was used to spray the pesticide. While spraying the highest part of the plant, they looked up to the end of the canopy to make sure that this part was really sprayed (Figure 3). The average spraying period per plant was 16.5 ± 2.5 seconds.

After completing the application the two volunteers did not experience any symptoms of pesticide poisoning. However, chemical analysis of the urine, blood and tissue papers indicated strong exposures upon application of the pesticide. Results of the exposure study also revealed that higher concentration used by the volunteer resulted higher residue levels of the pesticide on the analyzed samples. The residues of pesticide in urine and blood of the volunteer applying 1 ml increased 6.5 and 1.1 times, respectively while those of that 3 ml increased 10.3 and 1.5 times, respectively (Table 3).

Figure 3. Common way of farmers to spray pesticides on black pepper plants on Bangka Island, Indonesia.

Table 3. Chlorpyrifos residue levels in urine, blood and tissue paper attached to the clothes of volunteers before and after spraying 100 pepper plants with chlorpyrifos according to common practices.

Volunteers	Concentration of chlorpyrifos	Sample	Insecticide residue (µg g^{-1})	
			Before application	After application
Volunteer A.	1 ml l-1	Urine	0.0002	0.0013
		Blood	0.0015	0.0016
		Paper	nd	0.2044
Volunteer B.	3 ml l-1	Urine	0.0003	0.0031
		Blood	0.0015	0.0023
		Paper	nd	0.2219

nd = not determined

The difference in chlorpyrifos level between the tissue papers on the farmers spraying 1 and 3 ml l^{-1} was only small i.e. 1.08 times. It gave the impression that the slight different levels of protective clothing wore by the volunteers might not influence the actual exposure to pesticide since the volunteers might be exposed mainly through inhalation. The pre sprayed residues detected in their bodies probably resulted from earlier regular exposures since their used to spray pesticides to control pests in botanical gardens of the RIISICRI, in where this farmer exposure study was also conducted.

Gas chromatographic analysis on the pepper berries obtained from a local market and 2 exporters showed that the residual levels were below the standard maximum residue limit (MRL) on food as generally defined by [51]; [52]. In all cases and often the residues bellowed the limit of detection (Table 4).

Table 4. Residues of three synthetic insecticides frequently used by farmers on pepper berries on Bangka Island

Source of samples	Lambda-cyhalothrin ($\mu g\ g^{-1}$)		Fenthion ($\mu g\ g^{-1}$)		BPMC (2-sec-butylphenyl methylcarbamate) ($\mu g\ g^{-1}$)	
	MRL	Residue	MRL	Residue	MRL	Residue
Exporter 1		0.0192		0.0043		0.0110
Exporter 2	0.02	<dl	0.05	0.0046	0.05	<dl
Local Market (Belinyu)		<dl		0.0041		<dl

<dl = below limit of detection. The berries were sampled from a local market and 2 big local exporters. MRL stands for Maximum Residue Limit as defined by the Pesticide Residue Committee [53] and Staatscourant [52].

Table 5. Exposure toxicity ratios (ETRs) as calculated by the PRIMET model for the aquatic ecosystem and terrestrial below ground invertebrates.

Active ingredient	Application (g ha^{-1})	ETRsoil	ETRwater
Permethrin	10	nd	260a
Cypermethrin	46	0.0084	360a
Beta-cyfluthrin	25	0.0300	170a
Fenthoate	96	nd	nd
Lambda-cyhalothrin	25	0.0580	3
Chlorpyrifos	400	0.0068	1900a
Carbosulfan	900	nd	20
Isoprocarb	1000	nd	nd
Cartap hydrochloride	1000	nd	2400a
Carbaryl	2125	0.4500	900a
Paraquat dichloride	828	0.0340	0.015

ETR values below 1 indicate absence of risks, between 1 and 100 small potential risks and above 100 large risks. nd indicates that the ETR was not determined because toxicity data were not available. a Represents large risks

Table 5 provides the results of the Environmental Risk Assessment in terms of ETRs for the aquatic and terrestrial compartments. Meanwhile realistic worst case assumptions were chosen, large potential risks were calculated for the aquatic ecosystem, while risks were absent for the terrestrial below ground invertebrates. For the aquatic environment the highest potential risks were indicated for the insecticides cartap hydrochloride, chlorpyrifos and carbaryl, while lower ones were calculated for carbosulfan and lambda-cyhalothrin insecticides and a paraquat dichloride herbicide

2.4. Discussion

The baseline study reveals that problems on the usage of pesticides in pepper plantations on Bangka Island seem to arise predominantly from unwise use of pesticides rather than from the toxic nature of the pesticides itself. This includes too high concentrations being applied, using single brand of synthetic pesticide continuously, poor application technology and rarely use standard protective equipments,

The study presented in the present paper indicated that farmers were severely exposed to pesticides since 21 respondents (17.9%) had experienced acute pesticide poisoning, with the common symptoms were headache, fatigue, dizziness, and diarrhoea. This condition may happen since during spraying pesticides, farmers are rarely wearing standard protective equipments. The interview revealed that most farmers do not like to wear the standard protective equipments because they are extremely inconvenient and uncomfortable to use, especially under high temperature field conditions. According to Rainbird and O'Neill [54], these senses are exacerbated by the higher human energy expenditure associated with carrying and operating a knapsack sprayer which heated their bodies causing sweating during pesticide application. Moreover, the equipments are sometimes not completely available on the market, costly, poorly maintained, as well as not designed for tropical climates. Therefore, the workers themselves probably are at the greatest risk of pesticide poisoning because of their close contact with concentrated forms of the toxic substances. Wilson and Tisdell [55] pointed out that too high exposure to pesticides was common in developing countries with each year tens of thousands of farmers being affected by exposure to pesticides. Little et al [56] performed a farmer survey in Thailand indicating significant health problems related to pesticide use were perceived by farmers. Headaches, dizziness and vomiting are also the most common symptoms thought to be linked to pesticide exposure. Konradsen et al [57] estimated that 3 million cases of pesticide poisoning occur worldwide annually with 220 000 deaths.

The volunteers in this study only sprayed 100 plants at 3ml l^{-1}, but still the residue levels of chlorpyrifos increased up to 10 times in urine (0.0003 to 0.0031 $\mu g\ g^{-1}$) and 1.5 times in blood (0.0015 to 0.0023 $\mu g\ g^{-1}$). In the real life situation the exposure to pesticides would be much higher than that of in these volunteers, since 32% of the farmers had more than 1000 plants. The highest exposure is to be expected for the agricultural workers i.e. 5 respondents (4%) without own plantation, who are applying pesticides very intensively. Exposure may be via inhalation of sprayed droplets (spray drift), from dermal exposure, either directly (during spraying or preparation of the formulation) or via wetted clothes and/or from ingestion during smoking or eating before washing [58)]. This can result in acute or chronic illnesses [59)] such as dermatitis [60)], as well as in physical and mental problems including anxiety, irritability, loss of memory and depression, which can lead to suicide [61)]. Interviewing of the volunteers revealed no symptoms experienced after application of the pesticide indicating that the exposure levels would be still relatively low. Studies of the [62)] demonstrated that 28 single daily doses of chlorpyrifos administered at 0.3µg g^{-1} day-1 produced no measurable cholinesterase changes or adverse clinical symptoms.

Sampling procedures to select study areas were based on data collected from provincial and district offices. Meanwhile, respondents were directly chosen in their fields located in the forests without considering economic status, age or size of pepper plantation. This limitation however could bias the finding since these factors might influence attitude of farmers in using pesticides. However, since the island was isolated, the education level of the workers was relatively low (graduated only from elementary to high schools), and pest control strategies were inherited from their parents the bias would not be so high.

Chemical analysis on the berries demonstrated that consumers were not at risk from pesticide residues since the levels of the analyzed synthetic pesticides on the berries were below Maximum Residue Limit (MRL) values for food in general as defined by [51, 52)]. Moreover, since this spice is used in relatively small quantities a relevant exposure via this product is not likely to occur especially compared to the risk of pesticide residues on daily food items that are consumed in larger quantities. Carcinogenic or reprotoxic effects are likely not occur as residue levels of the major pesticides tested were below the MRL values.

In this study, applying at theoretical model for calculating environmental impact of pesticide use, it could be demonstrated that pesticide use poses serious potential risks to the aquatic environment, if aquatic ecosystems are present adjacent to the treated fields (Table 5). [63)] reach the same conclusion for the use of pesticides in Thailand, while [44)] provide similar results for China and Vietnam. This indicates that the environmental side-effects of pesticide use receive too little attention in South-East Asia. This contamination of the aquatic ecosystem might not only harm the ecological integrity of the water, but also the livelihoods of local people in terms of reduced (drinking) water quality, reduced productivity (e.g. fish kills, effects on cattle that uses surface water as drinking water). Poor people are expected to be disproportionately affected by any deterioration in the environment [64)], and it is therefore important for ensuring the future availability of clean water in Asia to predict the effects of intensified agriculture on the biodiversity and quality of fresh water. It is, however, essential for a true estimation of risks that the results from this preliminary ecological risk assessment will be validated using chemical measurements, bioassays and bio-monitoring (the TRIAD approach, [65)]).

Though the organophosphates and the carbamates have a high environmental degradability and water solubility which both strongly reduce the risk of bioaccumulation, these chemicals still pose a threat to the local environment (Table 5.). Direct spraying exposure could kill wildlife and natural enemies of pest species living around the treated plants. In addition using the same pesticides all the time would stimulate the development resistance of the pests. Both factors might results in an outbreak of secondary pests [66)]. The study of [67)] provided evidence of the development of resistance of some cotton's insect pests in India i.e. *Helicoverpa armigera Hubner., Pectinophora gossypiella* Saunders., *Spodoptera litura* Fab., and *Earias vitella* Fab., to cypermethrin, chlorpyrifos, and endosulfan while *Bemisia tabaci* Genn. became resistant to cypermethrin after improper application of pesticides over the past two decades. Washing tanks in the river and throwing empty pesticide containers away in the forests are other causes for possible health risk for people, aquatic organisms and other living creatures in the area.

In general, this study indicates that local workers make unwise use of pesticides. To avoid further exposure of the farmers and the environment, they should be trained in why and how to use pesticides safely under tropical conditions [68)]. In addition, the current promotion of the Integrated Pest Management (IPM) control strategy including reduction of the application of synthetic pesticides to occasions when such pesticide use is necessary [69)]. IPM has successfully been applied to control the rice brown plant hopper, *Nilaparvata lugens* Stal. causing Indonesia to remain the world's largest rice importer for years [70)]. After applying the IPM strategy this country save more than \$100 million yr^{-1} by phasing out 85% pesticide subsidy between 1986 and 1989 while rice yields increase though average pesticide applications/season fall from over 4 to about 2.5 times [71)].

IPM also promotes the use of botanical pesticide to replace the synthetic ones. [72)] explained that the use of botanical pesticides is a strategy particularly helpful in reducing current environmental and health concerns because botanical pesticides are generally less persistent and therefore can be applied selectively after which they disappear. Therefore the use of botanical pesticides protects diversity and prevents the build-up of toxic residues in food chains and ecosystems. However, [34)] point out that the development of the pesticide needs further studies aiming at better characterizations of their toxic potencies, improved standardization of the quality of raw materials, and better definition of their formulation before their use can be implemented in industrialized and developing countries.

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Chapter

3

Nematicidal Activity of Plant Extracts against the Root-Knot Nematode, *Meloidogyne incognita*

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Abstract

Nematicidal activity of extracts from plants was assayed against *Meloidogyne incognita.* In the laboratory assays extracts from tobacco (*Nicotiana tabacum* L), clove (*Syzygium aromaticum* L), betelvine (*Piper betle* L), and sweet flag (*Acorus calamus* L) were most effective in killing the nematode, with an EC_{50} that even was 5-10 times lower than the synthetic pesticides chlorpyrifos, carbosulfan and deltamethrin. The shapes of the dead nematodes differed in a characteristic way, and groups of pesticides and plant extracts could clearly be distinguished based on this phenomenon, which may be an indicator for the modes of action of the tested pesticides. In the green house bioassay clove bud and betelvine were tested as mulch. Experiments revealed that the total number of nematodes on the clove bud only was 7% of that for the controls and did not differ significantly from that of the recommended synthetic pesticide carbofuran. The application of clove buds as a botanical pesticide for future use against nematodes is highly promising since clove is the 6th major plant grown in this region, but the market value of clove has decreased sharply over the last years.

Key words: Black pepper, botanical pesticides, *Meloidogyne incognita*, mode of action, nematicide.

3.1. Introduction

Root-knot nematode, *Meloidogyne incognita* Kofoid and White (Chitwood) (Tylenchida: Heteroderidae) constitutes a major group of plant-parasitic nematodes affecting the quantity and quality of the crop production in many annual and perennial crops. Infected plants show typical symptoms including root galling, stunting and nutrient deficiency, particularly nitrogen deficiency [4)].

On Bangka Island, nematodes are considered to be one of the major problems in black pepper cultivation. In 2003, 4.900 ha of the total of 52.468 ha of pepper plantations were severely infected by this pest organism [50)]. Although, there is no information about the exact impact of nematode infection on the loss of pepper production, it is clear from visible inspection that severely attacked plants have a reduced vitality, produce less fruits, and which finally will die. [6)] informed that the yield losses of cotton production caused by *M. incognita* in 2002 were estimated to be between 18.0-47.3%. Therefore the presence of this pest in plantations has to be controlled.

The population of plant-parasitic nematodes in the field can be minimized through several approaches such as using natural enemies [73, 74)], enhancing cultural practices [75)], cultivating resistant cultivars [76)], and applying pesticides [77)]. Since the 1950s, however, farmers have relied mainly on synthetic pesticides rather than on other approaches. This sometimes results in excessive and unsafe use of synthetic pesticides [11)]. Therefore, it has become an important issue to find alternative control strategies, which are as effective as synthetic pesticides, safer to farmers, consumers, and the environment and relatively easily available at low price [78)]. One of possible techniques is the utilization of pesticides from plant origin, known as botanical pesticides [79)]. These pesticides are generally considered to be non-persistent under field conditions as they are readily transformed by light, oxygen and micro-organisms into less toxic products. Therefore no residues are expected on the products or in the environment [80)].

The study reported in the present paper is part of a larger project in which 17 plant species, selected based on their availability and potential use as botanical pesticide, are further tested for this purpose. Of 15 of these 17 plant species there are indications they may have some nematicidal potency. In the present study we evaluate the toxic potency of extracts from these 17 plant species against *M. incognita.* The results will be compared to that of some often used or advised synthetic pesticides, namely chlorpyrifos (the organophosphate) [81)], carbosulfan (the carbamate) [82)] and deltamethrin (the pyrethroid insecticide) because it is one of the major pesticides used by farmers on Bangka Island to control pests of the black pepper [11)]. The 2 most potent extracts subsequently are assayed in a green house experiment to evaluate effectiveness of their raw materials applied as a mulch to control the nematode attacking roots of the pepper plant.

3.2. Materials and Methods

3.2.1. Chemicals

The synthetic pesticides used were chlorpyrifos 200 g l^{-1}, carbosulfan 200 g l^{-1}, deltamethrin 25 g l^{-1}, and carbofuran 3G, purchased from the agro-chemical shop, Sarana Tani in Bogor, Indonesia. DMSO (99.9% pure for spectroscopy from Acros Organics), was supplied by Sigma Aldrich (Zwijndrecht, The Netherlands), Tween 80 (synthesis grade), acetone (100%, analysis grade) and ethanol (absolute, analysis grade) from Merck KGaA (Darmstadt, Germany) were supplied by VWR (Amsterdam, The Netherlands).

3.2.2. Preparation of the plant extracts

All plant materials were obtained from the experimental gardens of the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI) and extracted in the post harvest laboratory of the Institute. The 17 plant species and part of the plant used for extraction are presented in Table 6. The extraction procedures of the plant materials were based on the method described by [83)], except for cashew. In short 1kg material was dried in the sun for 4-5 days then grinded in a hammer mill (Reisch Mühle made by Karl Kolb (Dreieich, Germany)) using 3 mm grinders. To the 1 kg powder 5 l of ethanol (96%) was added followed by 3 hours mixing at 500 rpm using an electric mixer made by Karl Kolb (Dreieich, Germany). Subsequently, the mixture was left standing overnight in the dark at 28 ± 1ºC to allow further extraction of the active ingredients. After this, the mixture was filtered using Whatman no 91 filter paper and the residues were soaked and shaken again in 1 liter of ethanol for 2 hours. After that the solution was filtered again over a new filter and the first and second filtrate were mixed and concentrated using a rotavapor at 45°C for approximately 3 hours until all ethanol was removed and only oils were left. The extracts were transferred into brown glass bottles and stored at -4°C. Only cashew nut was treated differently as cashew nut shell liquid (CNSL) was prepared by pressing the shell of the cashew seed in a manual presser made by the post harvest division of the IMACRI, after which the liquid was collected and stored in a brown glass bottle. On the following week about 10 ml of each extract were poured into 20 ml of glass bottles and they were transferred to and stored at -20°C in the laboratory of the Section of Toxicology of Wageningen University until further use.

3.2.3. Laboratory exposure of nematodes

The laboratory experiment was conducted in triplicate in the Sub Department of Nematology, Wageningen University, the Netherlands. The tested nematode species, *M. incognita*, was harvested according to the method as described by [84)]. In short, roots of about 3 months old tomato plants previously infected with the nematode were washed in fresh tap water. After that the roots were cut into 1-2 cm length and put in a round filter container then gently were put in the funnel which had been placed in a mist chamber. Active nematodes will pass through the filter and sink to the bottom of the funnel stem. On the following 4 days nematodes can be harvested and used for the experiment. The average density of nematode juveniles in the suspension thus prepared was about 1750 ml^{-1}.

The pesticide stocks were made in 1 ml glass vials by diluting the extracts in a solvent mixture of DMSO:Tween 80:Acetone = 1:2:3. In a first pilot study the maximum tolerated total solvent concentration was determined and this should not exceed 5% to avoid unspecific toxicity. The test concentrations were made by adding 40µl of the plant extract or the synthetic pesticide stocks to 460 µl of fresh tap water in a 12-well plate. This so-called mixing-plate was gently shaken by hand for about 2 minutes to allow the pesticides to mix properly. After that, 150 µl of the solution was transferred into 24 well plates, the test plate. Next, 90 µl of the nematode suspension containing approximately 150 juveniles was added into that well and gently mixed for another 2 minutes. The solution then was kept standing overnight at 24ºC which after 24 hours the dead and alive nematodes were counted to evaluate the mortality rate. In a second pilot study a range-finding was performed to determine the rough toxicities of the pesticides in a single final concentration of respectively 5 mg extract of plant extract ml^{-1} water and 31.5 mg technical mixture of synthetic pesticide ml^{-1} water. In these stock solutions, however, the visibility of the nematodes was not enough. Therefore the nematode solution was washed to make the nematodes completely visible. Washing was done by first adding 0.5 ml of fresh water to the 24 well plates containing exposed nematodes, letting all nematodes settle again on the bottom of the well during 3 minutes, and carefully removing 0.5 ml again using a micro pipette. This procedure was repeated 3 times. In order to evaluate a possible recovery effect, the observation of the mortality of the nematodes was conducted twice during the pilot study. The first time was conducted immediately after washing the second time that of approximately 6 hours after the first observation. The mortality of the treated nematodes was determined using a stereo microscope with 10-fold magnification. Nematodes were considered dead when no movement was observed during two seconds even after mechanical prodding. As no recovery of nematodes was observed, this was not further studied in the final experiment. Washing to dilute botanical extracts before counting the nematodes also was not needed, as in the pilot experiment the dead nematodes were found to have a specific shape, defined as either straight (I-shape), bent (banana-shape), sigmoid (Σ-shape), and curly (∞-shape) which can be used to determine the death or live nematodes. These shapes were recorded in the final experiment. In the final experiments all pesticides were tested in at least 5 concentrations including a solvent control. The lethal concentrations LC_{20}, LC_{50} and LC_{90} were expressed as mg extract or technical mixture ml^{-1} water.

3.2.4. Green house experiment

The green house experiment was conducted at The Indonesian Medicinal and Aromatic Crops Research Institute, Bogor. Nematodes for inocula were collected from the roots of pepper plants which were grown in the Botanical Garden of the Bangka Belitung Assessment Institute for Agricultural Technology, and which were heavily attacked by the root-knot nematode, *M. incognita*. The nematodes were harvested according to the same method as in the laboratory experiment as described by [84)].

Inoculation of 6 months old pepper plant grown in a 2 liter pot containing sterilized soils, was conducted by pouring 10 ml of water containing 1000 nematode juveniles onto the soil surface. One week after the inoculation in which to let the nematodes infest roots of the pepper plants, 10 g of carbofuran 3%, 20 g of ground clove buds, or 60 g of dried betelvine leaves were applied evenly on the soil surface. Control consisted of pots without additional application. The experiments were performed with 10 replicates. Every pot was watered three times a week with about 350 ml of fresh water. Two months after mulching the nematodes present on the roots of the treated plant were collected and counted. Collection was conducted according to the method as described by [84)]. As much as 1 ml of 40 ml solution containing collected nematodes which had been homogenized using magnetic stirrer then was sampled using 1-ml micro pipette. The solution then was put in to the 1-ml Matsunami micro slide glass and the nematodes were counted using a compound microscope under 100x magnification.

3.2.5. Data analysis

The mortality rates of the nematodes in the exposure groups (P_O) were corrected for the mortality in the solvent controls (P_C) using Abbott's formula: P_T (%) = [100 x (P_O-P_C)P_C^{-1}] [85)]. The corrected mortality (P_T) was plotted against the pesticide concentration and fitted using Slide Write Plus 6.1 (Advanced Graphics Software Inc.) to determine the LC_{20}, LC_{50} and LC_{90} values. Because the log scale was used for plotting the data, the control data were plotted as a concentration 100 times lowers then the lowest test compound concentration. The method of 95% LSD intervals was conducted for the means, and analysis of variance (ANOVA) and the least significant difference (LSD) test using SAS program was used to compare the means of the bioassays. Data were transformed into $\sqrt{x}+0.5$ since some of the data were zero.

3.3. Results

The yield of the extraction procedure varied between 4% (cashew, pressed) and 22% (clove) (Table 6). In addition to clove also vetiver (14%), patchouli (12.4%) and castor bean (12%) had relatively high yields of more than 10%. The density of the concentrated extract (expressed as mg ml^{-1}) indicates a relatively oily content of the citrosa and pyrethrum extracts (0.68 and 0.75 mg ml^{-1} respectively), while the others had densities between 0.86 and 1.06 mg ml^{-1}.

Table 6. The botanical species and plant parts (sources) that were extracted, the extraction yield as percentage (mg 100mg^{-1}) relative to the original (dried) plant material and the density of the final product.

Scientific name	Common names	Source of materials	Extraction rate (%)	Density (g ml^{-1})	Ref.
*Syzygium aromaticum*L*)	Clove	Bud	22.2	0.91	13)
Nicotiana tabacum L*)	Tobacco	Leave	8.1	1.07	86)
Piper betle L*)	Betelvine	Leave	8.6	0.96	87)
Acorus calamus L*)	Sweet flag	Rhizome	6.5	0.92	88)
Chrysanthemum cinerarieaefolium L*)	Pyrethrum	Flower	9.6	0.75	89)
Cymbopogon nardus L*)	Citronella	Leave	6.2	1.06	90)
Derris elliptica Benth*)	Tuba root	Root	6.3	0.96	91)
Azadirachta indica L*)	Neem	Seed	5.1	1.61	92)
Piper nigrum L*)	Pepper	Berries	8.6	1.03	87)
Andropogon zizanioides L*)	Vetiver	Root	14.2	0.89	90)
Richinus communis L*)	Castor bean	Seed	11.9	1.04	93)
Annona muricata L*)	Graviola	Seed	9.6	0.85	94)
Cymbopogon citratus L*)	Lemongrass	Leave	9.5	0.86	95)
Anacardium occidentale L*)	Cashew	Seed	4.1	1.00	96) 97)
Pelargonium citrosa Van Leenii**)	Citrosa	Leave	9.7	0.68	98)
Pogostemon cablin Benth	Patchouli	Leave	12.4	0.89	-
Pachyrhizus erosus L	Yam bean	Seed	5.0	0.94	-

*) = known contains nematicidal properties **) = predicted has nematicidal effect.

In the pilot study at 5 mg extract ml^{-1} water, tobacco, clove and betelvine were found to be highly toxic to the nematodes, killing more than 80% of the nematodes while the others gave quite low mortality values (Table 7). Based on these findings, these plant extracts were divided into 3 main groups i.e. highly toxic (>80% mortality), consisting of clove, tobacco and betelvine, slightly toxic (10-20% mortality) consisting of sweet flag, pyrethrum, and citronella and not toxic (<10% mortality) consisting of the rest of the extracts tested. The concentration applied for the synthetic pesticides (31.5 mg technical mixture ml^{-1}, equivalent to 6.3 mg active ingredient of chlorpyrifos and carbosulfan and 0.8 mg of deltamethrin ml^{-1} water) was very-moderately toxic killing 40%-93% of the nematodes (Table 7). Therefore, to find the LC_{50} values chlorpyrifos was tested at 0, 4, 13, 22, and 31 mg technical mixture ml^{-1} water, and the other two that of at 0, 13, 22, 31, and 40 mg technical mixture ml^{-1} water.

Table 7. Mortality (%) and lethal concentrations (LC_{20}, LC_{50} and LC_{90} as mg.ml^{-1}) of M. incognita after 24 hours of exposure to botanical extracts or synthetic pesticides via the aquatic medium.

Tested compounds	Pilot study (Mortality (%) ± SD)	Final experiment Lethal Concentrations (mg ml-1)		
Plant extracts	Tested in 5 mg ml-1	LC20	LC50	LC90
Clove	98 ± 2.3	2.8	3.9	4.9
Tobacco	94 ± 3.1	1.3	1.9	3.6
Betlevine	83 ± 1.2	1.2	3.0	5.2
Sweet flag	17 ± 5.1	4.9	11.3	18.7
Pyrethrum	13 ± 3.0	8.9	> 19.2	> 19.2
Citronella	10± 3.3	5.7	> 19.2	> 19.2
Tuba root	9 ± 5.1	8.6	> 19.2	> 19.2
Neem	8 ± 3.0	> 19.2	> 19.2	> 19.2
Pepper	6 ± 9.5	> 19.2	> 19.2	> 19.2
Cashew	5 ± 3.3	> 19.2	> 19.2	> 19.2
Vetiver	4 ± 4.3	> 19.2	> 19.2	> 19.2
Castor bean	4 ± 5.0	> 19.2	> 19.2	> 19.2
Graviola	4 ± 5.0	> 19.2	> 19.2	> 19.2
Patchouli	4 ± 5.6	> 19.2	> 19.2	> 19.2
Lemongrass	4 ± 5.7	> 19.2	> 19.2	> 19.2
Yam bean	1 ± 5.9	> 19.2	> 19.2	> 19.2
Citrosa	2 ± 3.4	> 19.2	> 19.2	> 19.2
Synthetic pesticides	Tested in 31.5 mg ml-1			
chlorpyrifos	93 ± 3.4	8.7	19.4	30.7
carbosulfan	73 ± 4.4	12.7	25.3	36.1
deltamethrin	40 ± 4.4	20.8	> 40	> 40

The highly toxic group of plant extracts was further tested at concentrations of 0, 1.2, 2.4, and 4.8 mg ml^{-1} water, while the other groups were tested at concentrations of 0, 4.8, 9.6, and 19.2 mg ml^{-1} water. The extracts were not tested at a higher concentration as some of them (cashew, tuba root, and neem) did not mix adequately at these higher concentrations. In addition, those pesticides were not considered for possible future application as those would require great volumes of plant material, which would not result in a practical protocol for pesticide use. The results revealed that tobacco, clove and betelvine were highly toxic with LC_{50} values of 1.9 – 3.9 mg ml^{-1} water. Sweet flag was moderately toxic with an LC_{50} of 11.3 mg ml^{-1} water. The EC_{50} of tuba root, citronella and pyrethrum were not reached, but their LC_{20} was 5.7 – 8.9 mg ml^{-1} water. The remaining 10 extracts were not toxic to the nematode as the LC_{20} was not reached (> 19.2 mg ml^{-1}water) (Table 7). Representative dose response graphs from each of these groups of plant extracts were given in Figure 4.

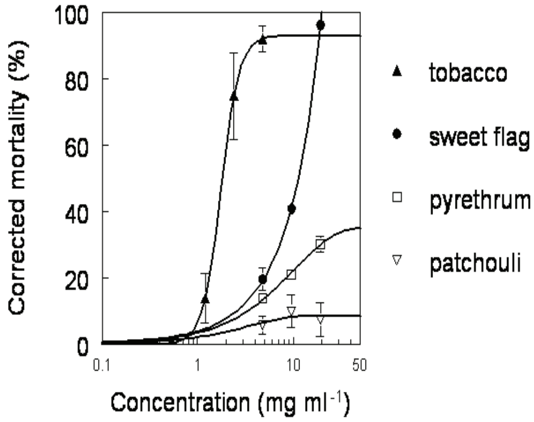

Figure 4. Examples of the dose response curves of highly, slightly and not acutely toxic plant extracts to the nematode *M. incognita.* Mortality was recorded after 24 hours of exposure via the aquatic medium. The experiment was performed in triplicate.

The synthetic pesticides chlorpyrifos and carbosulfan both fell in the slightly toxic group with an EC_{50} >19.2 mg ml^{-1} water and an LC_{20}.of 8.7-12.7 mg ml^{-1} water. Deltamethrin fell into the non-toxic groups with a LC_{20} > 19.2 mg ml^{-1}. This lower toxicity of the synthetic technical pesticide mixtures compared to the plant extracts can also be seen from the dose response curves (Figure 5).

When the dead nematodes were studied under the microscope it became apparent that they had either one of four very distinct shapes, namely: straight (I-shape), bent (banana-shape), sigmoid (Σ-shape), or curly (∞-shape) (Table 8, Figure 6). The dead nematodes from the control group mostly was straight (I shape) with only very few showing a bent (banana) shape. The characteristic shape of nematodes killed by tobacco and castor bean was curly (∞-shapes) with some bent and sigmoid shapes, which was similar to those killed by the acetylcholine esterase inhibitors chlorpyrifos and carbosulfan. The appearances of the nematodes killed by other plant extracts mostly followed straight or bent shapes, similar to those killed by the pyrethroid deltamethrin. The mortality and these characteristics were tested for consistence with the highest concentrations, and all pesticides yielded exactly the same results

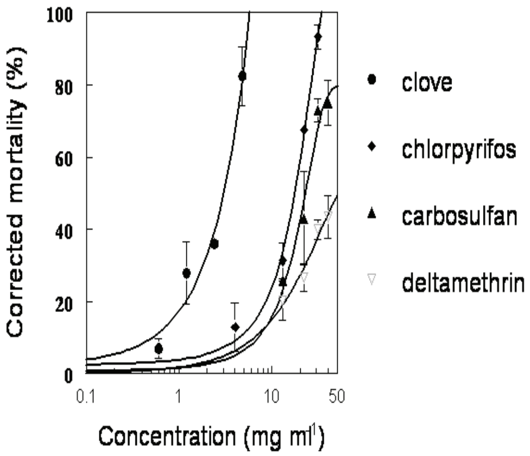

Figure 5. Acute toxicity of three synthetic pesticides to *M. incognita,* compared to that of the plant extract, clove. Mortality was observed after 24 hrs exposure. The experiment was performed in triplicates

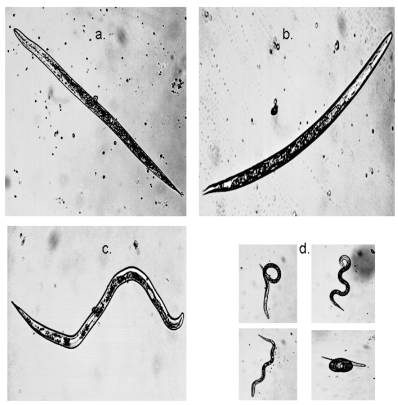

Figure 6. Characteristic shapes of dead nematodes: a. straight (I-shape), b. bent (banana-shape), c. sigmoid (Σ-shape), and d. curl (∞-shape). See table 8 for percentage relative occurrence of these shapes after exposure to the highest concentrations of plant extracts and synthetic pesticides.

Table.8. Relative occurrence (%) of characteristic shapes and percentage of relative occurrence among dead nematodes after 24 hrs of exposure to the highest concentration of pesticides via the aquatic medium.

Tested compounds	Shapes of dead nematodes (%)				Number of dead nematodes
	Straight (I-shape)	Bent (Banana-shape)	Sigmoid (Σ-shape)	Curled (∞-shape)	
Control	80	20	0	0	5
Clove	10	87	3	0	67
Tobacco	1	3	8	88	101
Betelvine	29	70	1	0	72
Sweet flag	25	73	2	0	101
Pyrethrum	0	100	0	0	13
Citronella	0	100	0	0	13
Tuba root	25	75	0	0	12
Neem	0	100	0	0	14
Pepper	37	63	0	0	8
Cashew	0	100	0	0	8
Vetiver	17	83	0	0	18
Castor bean	0	100	0	0	10
Graviola	17	79	4	0	36
Patchouli	21	79	0	0	14
Lemongrass	0	100	0	0	7
Yam bean	0	100	0	0	5
Citrosa	10	85	5	0	20
chlorpyrifos	1	13	11	75	103
carbosulfan	0	8	7	85	95
deltamethrin	12	87	1	0	38

In the green house experiment clove was 10 times more potent than betelvine in reducing the total number of nematodes in the roots 2 months after a single application. The number of nematodes in the root treated with the clove differed not significantly with that carbofuran. Although betelvine was able to reduce the infestation of the nematodes compared to control, this difference was not statistically significant. In addition, the number of infected plants treated with the clove bud was lower than that of the betelvine and control. There was no plant mortality among the clove and carbofuran treated plants, while this was 1 and 3 plants of the 10 in the betelvine-treated and control, respectively (Table 9.)

Table 9. The number of infected and dead pepper plants and the number of *M. incognita* presents 2 months after a single application of either as a mulch of clove or betelvine, carbofuran and a solvent control in a green house experiment. The plants were experimentally infected 7 days before exposure; n=10.

Treatments	Infected plants	# of death plants	# of nematodes ± SE per g roots*)
Control	10	3	335 ± 69.9b
Betelvine	8	1	274 ± 70.7b
Clove	5	0	23 ± 9.3a
Furadan	3	0	5.3 ± 5.1a

*) Means in the same column followed by the same letter do not differ significantly (P>0.05) in the LSD test.

3.4. Discussion

This study revealed that some plant extracts were highly toxic against nematodes in a laboratory exposure. One of which also was very effective in controlling infestation of nematodes into roots of the pepper plants during a 2 months semi-field testing. The *in vivo* laboratory study showed that tobacco (LC_{50} = 1.9 mg extract ml^{-1} water equivalent to 23.5 mg raw material ml^{-1} water), clove (LC_{50} = 3.9 mg extract ml^{-1} water equivalent to 17.6 mg raw material ml^{-1} water), and betelvine (LC_{50} = 3.0 mg extract ml^{-1} water equivalent to 34.9 mg raw material ml^{-1} water), were highly toxic for the parasitic root-knot nematode, *M. incognita*. Sweet flag was more moderately toxic (LC_{50} = 11.3 mg extract ml^{-1} water equivalent to 173.8 mg raw material ml^{-1} water). but still even more toxic than the three synthetic pesticides tested i.e. chlorpyrifos (LC_{50} = 19.4 mg technical mixture ml^{-1} water equivalent to 3.8 mg active ingredient ml^{-1} water), carbosulfan, a recommended pesticide to control this pest (LC_{50}= 25.3 mg technical mixture ml^{-1} water, equivalent to 5.1 mg active ingredient ml^{-1} water) and deltamethrin (LC_{50} = > 40 mg technical mixture ml^{-1} water equivalent to > 1 mg active ingredient ml^{-1} water).

This finding is very promising since farmers on Bangka Island indicated that currently there is no effective synthetic pesticide available to control this nematode in the field [11)]. The ineffectiveness of the pesticides used against the nematodes, may be because of the low concentrations the pesticides are applied to fight the nematode i.e. between about 0.001 – 0.004 mg technical mixture ml^{-1} water. These concentrations normally are used to control other pests such as tinged bug, *Dasynus piperis* China, stem borer, *Lophobaris piperis* Marsh and bug, *Diconocoris hewetti* Dist beetles [11)]. Our findings showed that the synthetic pesticides chlorpyrifos and carbosulfan would be effective if they could reach the nematodes in a concentration of at least 30 mg technical mixture ml^{-1} water. Carbofuran was not tested in our laboratory study since it could not be diluted adequately in the solvent, even after 10 minutes of sonification, making it impossible to compare exposure concentrations.

The observed characteristic differences in shape of the nematodes killed by pesticide-exposure was an interesting finding that might be useful as an indication to analyze the major mode of toxic action of the plant extracts of usually very complex composition. Meanwhile, according to [99] the nematicidal mode of action of plant materials still is not known. Our finding showed that the nematodes killed by the acetyl cholinesterase inhibitors chlorpyrifos and carbosulfan mostly had a curled shape (75-85%) while few of them had sigmoid (7-11%) and bent (8-13%) shapes.

The extract of tobacco, known to have acetylcholine esterase inhibiting action for human being [100], also induced curly (88%) and sigmoid (8%) shapes. This phenomenon can be explained because the neuromuscular junctions of nematodes are not fundamentally different, either structurally or functionally from the neuromuscular junctions of other animals [101] including mammals. Therefore, the shapes of the dead nematodes treated by the tobacco can be similar to those of treated by the organophosphate and carbamates pesticides.

The pyrethroid pesticide deltamethrin and the extract of pyrethrum, known for its pyrethroid-like action, resulted in dead nematodes that never had curly shapes but were mostly bent (banana-shape) (87-100%) and to some extent straight (I-shape) (0-13%) or very few of them showing a sigmoid shape (Σ-shape) (0-1%). The results shown in table 8 suggested that the mechanisms of toxicity behind the curly shape were related to that of the sigmoid shape, as their occurrence was related and they might just represent a gradual difference in occurrence of the toxicity. Based on the shapes of the dead nematodes we suggested that most of the extracts tested had a pyrethroid-like effect on the central nervous system of the nematodes. However, further assays in higher concentration or longer exposure period were helpful to be conducted to validate this finding since the mortality induced by most of the botanical extracts still was very low. We did not have any explanation yet for the clear relationship between shapes of the dead nematode in relation to the pesticide exposure. During the experiment it was observed that about five minutes after exposure of organophosphate and carbamate pesticides and a tobacco extract, nematode showed more active movement and most of them had formed curly shape and stable until they were die. On the other hand, those exposed by other treatments did not show specific shapes. Their appearances were similar as those in control.

Although the tobacco extract is the most toxic against the nematodes, it is not the best candidate to be applied in practice because of its high toxicity for mammals including man [102]. The other two highly toxic plants, clove and betelvine, are more prospective plants to be further developed into a botanical nematicide. Of these two clove gives the highest extraction yield (22.2%) followed by betelvine (8.6%). The extraction yield of the less toxic sweet flag only is 6.5% (Table 6). The application of ethanol for the extraction of plant extract as carried out in this study is not suitable for the farmers because it is too expensive for them while according to [103] high exposure of alcohol through inhalation often causing chronic obstructive lung disease. Therefore, easier and simpler preparation methods must be developed before a plant can be successfully introduced and applied as a botanical pesticide for the farmers. Two promising methods are application as an aqueous extract [104] and as an amendment of organic materials as mulch [105]; [106], [107]. In our study we choose for testing clove and betelvine as a mulch because mulching is believed to help control plant parasitic nematodes, as nitrate and ammonical nitrogen accumulated during decomposition of organic matters are toxic to plant parasitic nematodes [108]. The effectiveness of mulching to reduce the population of plant parasitic nematodes will be greatly enhanced when the mulch also contains toxic chemicals [109] such as the nematicidal compounds of clove. Amendment of organic plant materials also increases food sources which facilitates the population growth of bacterivorous nematodes (Rhabditidae and Cephalobidae), fungivorous nematodes [110] and predatory nematodes (Mononchidae) [111], which will also lower the population density of the plant parasitic nematodes through competition, antagonism or creating unfavourable conditions.

The green house experiment revealed that mulch from clove bud was very potent in suppressing nematode infestation in pepper plants. After 2 months of single application clove significantly suppressed the population of nematodes in the pepper plant roots. The dosages of clove and betelvine used during the green house experiment were based on the LC_{90} value of the laboratory bioassay, which was about 5 g extract l^{-1} solvent. This value was equivalent to about 20 g clove buds and 60 g dried betelvine leaves based on extraction yields of 22.2% and 8.6% respectively. The dosage of carbofuran was 10 g which was 1/3 of what would be recommended per plant in the field. This amount of carbofuran used was based on an assumption that the volume of the soil in the pot was assumed to be 1/3 of that in the field. The proposed dosage of clove bud for field application based on the greenhouse experiment therefore is 60g per plant.

Since clove is the 6th major cultivated plant on Bangka Island [50], this plant materials is prospective to be further developed as natural nematicide. Moreover, since price of the clove bud on the local market dropped from about 9 US\$ kg^{-1} in 2001 to about 3 US\$ kg^{-1} in 2005 [112] new additional uses of the clove bud as nematicide would be very welcome. To allow a successful introduction, practical information related to the use of plant materials to effectively control pests has to be developed and made available to the farmers. It is expected that application of clove, as a botanical pesticide will be adopted easily by local farmers on Bangka Island as in the past farmers in this region used plant materials as pesticides [11]. The results of the present study indicate that once a useful recipe is developed the use of clove can help to reduce the current intensive but not so effective used of synthetic pesticides against nematodes, with the connected risk for the human and environmental health.

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Chapter

4

Pesticidal Activity of 17 Plant Extracts against the Red Flour Beetle, *Tribolium castaneum*

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Abstract

The potency of 17 botanical extracts from Indonesian plants known to have insecticidal activity was assayed in 3 ways against adults of *Tribolium castaneum* (Herbst) (Coleoptera; Tenebrionidae). After topical application, pyrethrum extract was the most toxic followed by extracts from patchouli, cashew, tobacco, and sweet flag. When mixed with food (0.2% w w^{-1}), all extracts, except castor bean and yam bean extracts, were toxic upon oral exposure and acted as feeding inhibitor. At 28 days after oral exposure ten of the tested extracts killed more than 75% of the insects. In the dual choice bioassay pyrethrum and neem extracts had a strong repellent effect followed by clove, sweet flag, lemongrass and vetiver extracts. Extracts from citronella, castor bean, cashew, patchouli, and yam bean showed an attractant effect. It is concluded that pyrethrum, sweet flag, tobacco, clove and lemongrass are the most promising for future development and use as botanical pesticide since they were toxic upon both topical and oral application, and had a repellent effect against the tested model species *T. cataneum*.

Key words: Insecticidal activity, plant extracts, *Tribolium castaneum*.

4.1. Introduction

Concerns over health and environmental problems associated with the use of synthetic pesticides in agriculture have led to an intensification of efforts to find safe, effective, and viable alternatives for pest management [113)]. One of the approaches is to search for active compounds from plant materials that are able to control pests' population. These materials are expected to be more selective and less persistent, which will be beneficial for the environment, agricultural workers and consumers [72)]. In addition, these so-called botanical pesticides can be grown and produced locally.

Botanical products have been used by man since ancient times, especially in cultures with a strong herbal tradition [12)]. These products have also been studied for topical toxicity, antifeedant or repellent, attractant and fumigant effects as well as for inhibition of reproduction of many pest species [114)]. Although the mechanisms of action differ greatly and are not yet well understood, this widespread range of potencies, makes that nowadays botanical pesticides are being considered more often for their use in pest management strategies [33)]. Some plants grown in Indonesia that are known to have insecticidal activities are pyrethrum *(Chrysanthemum cinerarieaefolium* Trev*)*, sweet flag (*Acorus calamus* L), tobacco (*Nicotiana tabacum* L), and clove (*Syzygium aromaticum* L). There is however no report of a systematic comparative study of the potential of different plant extracts as botanical pesticides. The current study therefore was undertaken to investigate the bioactivity of crude extracts of Indonesian plants reported to have insecticidal properties and which can be easily found in the field. The following plants were selected.

Pyrethrum has been reported effective for the control of storage pests such as *Sitophilus granarius* (L) [17)], *Rhyzopherta dominica* (F) [26)] and *Tribolium confusum* (DuVal) [15)]. Since it poses low environmental risk, pyrethrum is an ideal pesticide for outdoor pre-harvest treatment [17)]. Sweet flag is another potent plant which is effectively used to control *Prostephanus truncatus* (Horn), the main storage pest in East and West Africa, [16)], and *Lasioderma serricorne* F, the most serious pest of tobacco, cereal grains and processed food in Korea [115)]. The plant is also effective against *Sithopylus oryzae* (L). a rice weevil in storages as well as against *Callosobruchus chinensis* (L) [116)] and *C. phaseoli* Gyllenhall, pests of stored beans [18)]. Pre-harvest application of tobacco solution can reduce infestation of the stored seeds of cowpea by *C. maculatus* (F). Applying tobacco at the podding stages reduces population of pod pest in the field such as *Clavigralla tomentoscollis* (Stat) and *Riptortus dentipes* (Fab.) [32)]. Clove has been known to have high repellent effect and feeding inhibition activities of storage pests like. *T. castaneum* (Herbst) and *S. zeamais* (Motsch) [27)] . Its repellent effect also has been reported to be able to control ecto parasites such as the the poultry mite *Dermanyssus gallinae* (De Geer) [19)] and the tick species, *Iodes ricinus* (L), parasitic on sheep, cattle and humans [20)].

Other promising Indonesian plants that might be used are patchouli (*Pogostemon cablin* Benth) [30], yam bean (*Pachyrhizus erosus)* [24], castor bean (*Richinus communis* L.) [29], black pepper (*Piper nigrum* L.) [117], neem (*Azadirachta indica* A Juss) [28], lemongrass (*Cymbopogon citratus* DC.) [118], betelvine, (*P. betle* L) [31], citronella, (*Cymbopogon nardus* L) [119], tuba root, (*Derris elliptica* Benth) [120], cashew (*Anacardium occidentale* L) [121], vetiver (*Andropogon zizanioides* L*)* [25], graviola (*Annona muricata L)* [122], and citrosa (*Pelargonium citrosa* Van Leenii) [123].

The present study aims to provide information about the bioactivity of crude extracts of these 17 plants. The study was performed with adults of the model insect pest species *T. castaneum* (Coleoptera: Tenebrionidae). This species was selected as a model species for black pepper pests because it can be easily reared under laboratory conditions. The tests of the present study in which the different botanical extracts were investigated for their activity against *T. castaneum* include mortality after direct topical application, mortality after dietary exposure and repellent effect when offered in a dual choice option using food with and without the plant extract. The results of this study provide insight in which botanical species and their extracts are most promising for further development of botanical pesticides against pests of black pepper.

4.2. Materials and methods

4.2.1. Preparation of the extracts

All plant materials (presented in Table 10) were collected from the experimental gardens of the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI). The extraction procedures were described before [11]. In short, the dried plant material was grinded in a hammer mill followed by extracting during 24 hours of the essential oils using ethanol (96%). After that the mixture was filtered using Whatman no 91 filter paper and subsequently the solution was concentrated using a rotavapor. The extracts were transferred into brown glass bottles and stored at -4°C and used for the assays within two weeks. It was expected that there was no significant biodegradation of the extracts during this period since based on a previous study, the pyrethrum based pesticide has been proven stable until 1 year stored in this condition [124].

4.2.2. Insects

The red flour beetle, *T. castaneum,* was collected from wheat flour sold in a Pasar Anyar, a local market in Bogor, Indonesia. The pest species was then reared in the Laboratory of the Plant Protection Division of the IMACRI and maintained at 29 ± 2°C, a relative humidity (r.h.) of 65-73 % and a photoperiod of 12 hours. Emerging adults of ± 2 weeks old weighing on average of 1.81 ± 0.22 mg were used for the experiments.

4.2.3. Bioassays

All botanical extracts and the synthetic pesticide, deltamethrin 2.5EC (supplied by Setiaguna Bogor, Indonesia) were diluted in a mixture of one part of DMSO (99.9% pure for spectroscopy from Acros Organics, supplied by Sigma Aldrich, Zwijndrecht, The Netherlands), with nine parts of acetone (80% Merck KGaA supplied by Setiaguna, Bogor, Indonesia). The extracts were assayed for their toxicity and repellent activity in the laboratory of the IMACRI using the following methods;

4.2.4. Topical application

A concentration range of the botanical extracts of 0, $5x10^1$, $1x10^2$, $2x10^2$, and $4x10^2$ µg ml^{-1} and of deltamethrin of 0, $3.2x10^{-3}$, $6.3x10^{-3}$, $1.3x10^{-2}$, $2.5x10^{-2}$, and $5x10^{-2}$ µg ml^{-1} were prepared and stored in brown glass bottles at -4°C. Aliquots of 1µl per insect were topically applied to the thorax of individual insect using a micropipette according to a method described before [125]. Three groups of insects were exposed, and each group was kept in a Petri dish containing 2 g of wheat flour and kept in a dark room at 29 ± 2ºC, 65-75% r.h. The controls were treated with the solvent only. Mortality was recorded at 1 hour after application and every 24 hours thereafter until 72 hours. Insects were scored dead when they did not move even after being gently touched with a wooden stick of ± 1mm diameter. The toxic dose was expressed as µg of extract per mg of insect calculated based on their average weight of 1.81 mg.

4.2.5. No choice bioassay

The no choice bioassay was performed according to a method previously described [126]. An aliquot of 2 ml acetone solution containing 5 mg of the test substance per ml was added to 5 g of wheat flour to reach a final concentration of 2 mg extract per gram food. Of the mixture 15 mg was placed in a Petri dish of 15 cm^2 and the acetone was evaporated overnight at ±29°C. Twenty four hours after the diet was prepared, groups of 20 insects were transferred to the Petri dishes in 3 replications. The blank exposure was prepared in the same way with solvent only, while the positive control was a group of insects kept without food. The mortality of the insects was recorded weekly until all insects from the positive control had died, which was four weeks after application.

4.2.6. Dual choice bioassay

The dual choice bioassay was conducted to measure the repellent effect of the extracts, and performed according to the method described before [126] offering treated and untreated diet in a Petri dish. The extract stocks were mixed with the diet at 2 mg extract per gram food via dilution in 1 ml of acetone, mixing with the food and subsequent evaporation of the acetone during 24 hours. The bioassay was performed in triplicate using 20 adults released in the middle of a 15 cm^2 petri dish. After 1 and 24 hours, the number of insects presents at the location of the treated (T) or control (C) diet was counted. The repellent index (RI) was calculated using the following formula: RI. = (C-T)/C+T) x100% [126]. Positive values indicate repellent effects and negative values attractant effects.

4.2.7. Data analysis

The mortality data (P_O) for the topical and the no choice bioassays was corrected for the mortality in the solvent controls (P_C) using Abbott's formula: P_T (%) = [100 x (P_O-P_C)P_C^{-1}] [85]. The corrected mortalities (P_T) on the topical bioassay were evaluated to find estimated lethal concentration (LC_{50} and LC_{90}) while those on the no choice bioassay were evaluated to find estimated lethal time (LT_{50}) using Slide Write Plus 6.1 (Advanced Graphics Software Inc.). The difference among data in the no choice and dual choice bioassays were analyzed using analysis of variance (ANOVA) and the least significant difference (LSD) using SPSS program with 95% of interval.

4.3. Results

4.3.1. Topical bioassay

The topical bioassay showed that extracts from pyrethrum, patchouli, cashew, tobacco and castor bean had a knock down effect, resulting in a sudden death of the treated insects following the extract application. Pyrethrum, patchouli, cashew, tobacco and sweet flag extracts were highly toxic against *T. castaneum* as they were able to kill 90% of the treated insects and showed LC_{90} values in the range of 0.006-0.237 µg extract mg^{-1} insect. Castor bean, clove and lemongrass extracts showed moderate toxicity as they were not able to reach LC_{90} values. The LC_{50} of those extracts were 0.100, 0.128, and 0.174 µg mg^{-1} insect, respectively. Some insects appeared to recover from the acute exposure since the LC_{50} or LC_{90} values increased with increased observation time (Table 10.).

Pyrethrum extract was the most toxic plant extract against the model pest species when applied topically with an LC_{50} value at 72 hours after application of 0.003 µg mg^{-1}. The LC_{90} value after the same observation period was 0.006 µg mg^{-1}. These values were about 400 and 230 times higher than respectively the LC_{50} (0.00001 µg mg^{-1}) and LC_{90} (0.000026 µg mg^{-1}) values of deltamethrin (Table 10). The LC_{50}'s of the other extracts after the same observation period were about 4500-17000 times higher than those of deltamethrin, while the LC_{90} values were about 2700-9200 times higher.

4.3.2. No choice bioassay

The no choice bioassay demonstrated that all extracts except castor bean and yam bean extract showed oral toxicity since most of the tested extracts caused 50% mortality 18-38 days after oral exposure. The mortality of the insects in the treated and untreated groups after 7 and 14 days did not differ significantly from that of the starved insects ($p>0.05$).

Table 10. Mortality of *T. castaneum* treated topically with 17 plant extracts and a synthetic pesticide at 29 ± 2^0C and 65-75% r.h. was expressed in LC_{50} and LC_{90} (µg extract mg^{-1} body wt). Mortality data were corrected for mortality observed in the solvent control. Observation was conducted at 1, 24, 48, and 72 hours after application.

Scientific names	Common names	LC50				LC90			
		1 h	24 h	48 h	72 h	1 h	24 h	48 h	72 h.
C. cinerarieaefolium	Pyrethrum	0.003	0.002	0.003	0.003	0.006	0.003	0.006	0.006
P. cablin	Patchouli	0.060	0.040	0.040	0.040	0.096	0.062	0.070	0.070
A. occidentale	Cashew	0.086	0.080	0.070	0.070	0.530	0.319	0.187	0.187
N. tabacum	Tobacco	0.176	0.200	0.147	0.140	0.230	0.215	0.209	0.202
A. calamus	Sweet flag	>	0.090	0.120	0.110	>	>	0.237	0.237
R. communis.	Castor bean	0.209	0.180	0.114	0.100	>	>	>	>
S. aromaticum	Clove	>	>	>	0.128	>	>	>	>
C. citratus	Lemongrass	>	>	0.179	0.174	>	>	>	>
P. nigrum	Pepper	>	0.140	0.130	>	>	>	0.400	>
C. nardus	Citronella	>	>	>	>	>	>	>	>
P. citrosum	Citrosa	>	>	>	>	>	>	>	>
P. betle	Betelvine	>	>	>	>	>	>	>	>
A. muricata	Graviola	>	>	>	>	>	>	>	>
D. elliptica	Tuba root	>	>	>	>	>	>	>	>
A. indica	Neem	>	>	>	>	>	>	>	>
A. zizanioides	Vetiver	>	>	>	>	>	>	>	>
P. erosus	Yam bean	>	>	>	>	>	>	>	>
Deltamethrin	Positive control	0.000008	0.000008	0.000008	0.00001	0.000014	0.000017	0.000026	0.000026

> Estimated LC_{50} and LC_{90} values were beyond the maximum tested exposure concentration (> 0.221 µg extract mg^{-1} body weight)

Extracts from clove and sweet flag were the most toxic and mortality rates upon exposure to these extracts differed significantly from those of the control at 21 days ($p<0.05$). The highest mortality after 28 days was found for extract of sweet flag (100%) followed by extracts of pyrethrum (95%), clove (90%), graviola and citrosa (85%), neem (79%), tobacco, black pepper, and vetiver (75%), betelvine (71%), and citronella (65%). These mortalities did not differ significantly ($p>0.05$) from those of the starved insects (90%) (Table 11).

Table. 11. Average mortality of *T. castaneum* in a no choice bioassay exposed to 2 mg extract gram^{-1} food. Mortality was scored at 7, 14, 21 and 28 days following oral exposure. Mortality data on the treated food were corrected for mortality observed in the negative control. Negative numbers indicate that the insects lived longer than the starved insects.

Plant Extracts	Days after application (daa) (Means ± SD)				LT50 (days)
	7	14	21	28	
No Food	0 ± 0 a	0 ± 0 a	4 ± 17 cde	85 ± 14 ab	27
Pyrethrum	3 ± 6 a	10 ± 10 a	18 ± 37 bcd	95 ± 8 a	25
Patchouli	3 ± 6 a	10 ± 10 a	19 ± 23 bcd	55 ± 15 cd	27
Cashew	10 ± 17 a	17 ± 29 a	34 ± 18 abc	45 ± 15 d	38
Tobacco	0 ± 0 a	10 ± 10 a	19 ± 17 bcd	75 ± 23 abc	26
Sweet flag	0 ± 0 a	23 ± 6 a	50 ± 25 ab	100 ± 0 a	21
Castor bean	0 ± 0 a	0 ± 0 a	-16 ± 8 f	-15 ± e	>28
Clove	0 ± 0 a	30 ± 26 a	62 ± 17 a	90 ± 9 ab	18
Lemongrass	3 ± 6 a	23 ± 12 a	27 ± 14 bc	44 ± 37 d	34
Black pepper	10 ± 17 a	10 ± 17 a	26 ± 16 bc	75 ± 7 abc	23
Citronella	3 ± 6 a	10 ± 0 a	19 ± 6 bcd	65 ± 7 bcd	27
Citrosa	0 ± 0 a	7 ± 6 a	11 ± 11 cde	85 ± 1 ab	27
Betelvine	3 ± 6 a	10 ± 0 a	27 ± 6 bc	71 ± 13 abcd	25
Graviola	0 ± 0 a	13 ± 12 a	30 ± 23 abc	85 ± 14 ab	24
Tuba root	3 ± 6 a	7 ± 12 a	19 ± 6 bcd	45 ± 4 d	29
Neem	0 ± 0 a	10 ± 10 a	15 ± 26 cde	79 ± 18 abc	26
Vetiver	0 ± 0 a	7 ± 12 a	23 ± 10 bc	75 ± 7 abc	25
Yam bean	0 ± 0 a	0 ± 0 a	-12 ± 1 ef	-21 ± 26 e	>28

LT_{50} = interpolated time until 50% of the animals are dead. >28 indicates the LT_{50} was not reached within 28 days. Means in the same column followed by the same letters do not differ significantly ($P>0.05$) in the LSD test

4.3.3. Dual choice bioassay

The results of the dual choice bioassay expressed as repellent index (RI) of the extracts at 24 hours after application were divided into 4 categories; The first group consist of extracts with a high RI (RI= 70-100%) and contains pyrethrum, neem, clove, lemongrass, sweet flag and vetiver extracts. The second group contains extracts with an intermediate RI (RI= 30-40%) including tobacco and graviola extracts. The third group contains extracts with a low RI (RI=3-20%) including extracts of black pepper, tuba root and citrosa. Finally the fourth group contains extracts that showed an attractant effect (RI= -2 - -28%) and includes citronella, castor bean, cashew, patchouli, and yam bean extract. The experiment also showed that extracts which had a relatively low RI tended to have RI values with higher standard deviation at both observation periods (Table 12.).

Table 12. Repellent index of the 17 plant extracts against *T. castaneum* as determined in a dual choice bioassay (average of 20 observations per group). The pesticides were tested in triplicate at 2 mg extract per gram food and repellent index was determined 1 and 24 hours after exposure.

Plant Extracts	Repellent index ± SD	
	1 hour	24 hours
Pyrethrum	93 ± 12 a	100 ± 0 a
Patchouli	-27 ± 16.4 f	-6 ± 46 de
Cashew	-24 ± 22 ef	-28 ± 50 e
Tobacco	53 ± 31 abcde	40 ± 0 abcde
Sweet flag	87 ± 23 ab	80 ± 20 abc
Castor bean	-20 ± 20 def	-7 ± 23 e
Clove	93 ± 12 a	87 ± 23 ab
Lemongrass	67 ± 58 abc	80 ± 20 abc
Black pepper	40 ± 57 abcdef	20 ± 47 abcde
Citronella	-3 ± 64 cdef	-16 ± 71 de
Citrosa	56 ± 36 abcd	8 ± 44 bcde
Betelvine	12 ± 66 bcdef	0 ± 65 cde
Graviola	18 ± 59 abcdef	35 ± 61 abcde
Tuba root	36 ± 67 abcdef	20 ± 81 abcde
Neem	60 ± 35 abc	100 ± 0 a
Vetiver	80 ± 0 ab	73 ± 12 abcd
Yam bean	-13.3 ± 23 cdef	-26.7 ± 12 e

- = Showed attractancy effect

4.4. Discussion and Conclusion

The results of the present study reveal that an alcoholic extract from pyrethrum followed by those of sweet flag, tobacco, lemongrass, and clove were the most potent extracts for use as a botanical pesticide against *T. castaneum*, the model pest selected for the present studies. These extracts were able to control the pest through contact toxicity, oral toxicity and repellent activity. Meanwhile, neem, vetiver, graviola, black pepper, citrosa, and tuba root extracts were moderately potent since they only were effective in controlling the pest through oral toxicity and repellent activity. Cashew and patchouli extracts also were moderately potent through contact toxicity and oral toxicity. Castor bean and citronella extracts were slightly potent through oral toxicity or repellent activity only (Table 13).

Table 13. Qualifications of the potency of the extracts in the 3 bioassays with *T. castaneum*.

Classes	Topical bioassay	No choice bioassay	Dual choice bioassay
	3= LC_{90} reached at 72 hrs 2= LC_{50} reached at 24 hrs 1= LC_{50} reached at 72 hrs	3= LT_{50} reached >18 days 2= LT_{50} reached >23 days 1= LT_{50} reached >28 days	3= RI >70% 2= RI >30% 1= RI > 3%
All round application			
Pyrethrum	3	2	3
Sweet flag	3	1	3
Tobacco	3	2	2
Spraying application			
Patchouli	3	2	0
Cashew	3	1	0
Storage application			
Clove	1	1	3
Lemongrass	1	1	3
Neem	0	2	3
Vetiver	0	2	3
Black pepper	0	3	1
Graviola	0	2	2
Citrosa	0	2	1
Not so effective			
Castor bean	2	0	0
Citronella	0	2	0
Betelvine	0	2	0
Tuba root	0	1	1
Yam bean	0	0	0

[1] Clove has been shown to be very effective as nematicide against root knot disease in the field

These assays showed that the mode of action of the plant extracts to control the treated pest differed among each other. However, most of the tested extracts except castor bean and yam bean extracts showed oral toxicity, killing insects that consumed the treated food. Interestingly citronella, cashew, and patchouli extracts, which acted as an attractant, also showed oral toxicity (Table 13). Hence the attracted pests finally would die after ingesting the treated food.

Citronella [20, 127, 128)], cashew [129)] and patchouli [30)] are known repellents to many insect pests such as *I. richinus, Amblyomma hebraeum* (Koch), *C. maculatus*, *Periplaneta americana* (L.) and *S. paniceum*. However, the present study showed that these extracts acted as an attractant to *T. castaneum*. As those extracts were orally toxic, as shown in the no choice bioassay, 45 to 55% of the attracted animals died after all. This may be due to interspecies variation of the insects causing differences in behavioural responses to the extract. Hence, some substances that repel one pest can even serve as an attractant or stimulant for other pests [130)].

Based on these experiments the most promising candidates for consideration as botanical pesticides to be applied in pepper plantations are the extracts of pyrethrum, sweet flag, and tobacco. These plant extracts were able to kill the treated pest through contact and oral toxicity and also acted as strong insect repellent. These extracts plus lemongrass and clove extracts may be used to control pests in storage and in the field. Using those plants as botanical pesticides in the field also may avoid infestation of pests before the pepper berries are being stored as infestation often begins in the field from where it is carried over to the storage [32)].

In addition to extracts from clove and lemongrass, neem, vetiver, graviola, citrosa and black pepper extracts (the latter for non-pepper applications) seem to be promising for future development and use as botanical pesticides to reduce infestation of stored crops and seeds by pests, since they induced oral toxicity and had repellent activity. The present study tested alcoholic extracts of the different pant varieties. Future application, however, may be through mixing either the plant materials [131)] or aqueous extracts into the storage products or by impregnating the bags or containers with the aqueous extracts of the plants [132)]. The aqueous extracts are cheaper than the ethanol extracts used in this study and therefore more suitable for use by farmers. Whether these aqueous extract are equally effective as the alcoholic extracts of the present study remains a topic for future investigations, but given the polarity of both media the results are expected to be reasonably comparable.

In addition to the above mentioned bioactivities, the raw material of clove bud has been shown to be very potent against the root-knot nematode, *Meloidogyne incognita*, one of the key pests of black pepper on Bangka Island, Indonesia [11)]. Since clove had a high repellent effect, application of the clove bud as mulch to control the nematode will also repel pests living on the treated plants. Furthermore, the other way around, drifts of the extract solution sprayed into the pepper canopies will reach the soil around the rooting areas, which indirectly also may reduce the presence of nematodes.

Based on our findings, the use of plant extracts in agricultural products presents a promising future development. . The increased consumer request in developed countries for organic products which are free from pesticide residues currently stimulates the interest in the use of botanical pesticides in agricultural production by exporting tropical countries [37)]. The use of botanical pesticides may be considered to be safe because the concentrations that remain on the stored products are expected to be neglectable since the pesticides are generally easily degraded through biodegradation [72)]. Practical considerations such as ease of growing the plants and safety for the applier and consumer also are important considerations when choosing the plant species and way of application of plants as botanical pesticides. Altogether the results of the present study identify alcoholic extracts from especially extracts of pyrethrum, sweet flag, tobacco, clove and lemongrass as promising candidates for the further development of future effective botanical pesticides.

Chapter

5

Laboratory and Field Studies on the Effectiveness of Botanical Extracts and a Newly Formulated Botanical Pesticide Formulation against Pests of Black Pepper

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Abstract

To reduce the dependence on the sometimes unwise use of synthetic pesticides in black pepper (*Piper nigrum* L.) plantations, the toxicity and repellency of ten botanical extracts was studied in 3 bioassays using *L. piperis*, a major pest species of black pepper. Based on the three most active extracts in these laboratory tests a new botanical pesticide formulation was defined which was subsequently tested in laboratory tests with 6 pest species of black pepper as well as in green house and field experiments. The laboratory bioassays with *L. piperis* showed that extracts from pyrethrum (*Chrysanthemum cinerarieaefolium* L), sweet flag (*Acorus calamus* L) and clove (*Syzygium aromaticum* L) were the most potent extracts and can be used as botanical pesticide because they showed the highest toxicity and/or repellent effect toward this selected model pest species. Based on these three plant extracts a new botanical pesticide formulation was defined with active ingredients being pyrethrin, ß-asarone, and eugenol. The newly developed formulation contained 1.8% pyrethrin, 7.8% ß-asarone, and 4% eugenol and was shown to be effective against 6 important pepper pest species including *Dasynus piperis* (China), *Diconocoris hewetti* (China), *Aphis craccivora* (Koch.), *A. gossypii* (Glover.), *Ferrisia virgata* (Cockerell.) and also against the aquatic insect, *Culex pipiens* (L.). In field experiments the formulation was able to control most pest

species of the pepper plants in the meantime being less toxic towards the 11 monitored species of natural enemies for known pest organisms such as caterpillars, aphids, moths, beetles than that of the recommended synthetic pesticide, deltamethrin. Furthermore, in field experiments it was revealed that within 9 hours after application the treated plants were recolonized again by ants and spiders, indicating a short degradation period of the formulation. Altogether it is concluded that the newly defined botanical formulation provides an effective and environmentally friendly alternative for controlling several pests of black pepper.

Key words: black pepper, botanical pesticide, clove, formulation, pests, pyrethrum, sweet flag.

5.1. Introduction

A common problem among agricultural workers in most developing countries is too high exposure to synthetic pesticides [55)]. In Bangka Island, Indonesia, synthetic pesticides have been applied intensively to control the three main pests of pepper plants (*Piper nigrum* L) namely a stem borer, *Lophobaris piperis* (China) (Coleoptera; Curculionidae), a green pepper berry bug, *Dasynus piperis* (China) (Hemiptera; Coreidae) and the bug *Diconocoris hewetti* (Dist) (Hemiptera; Tingidae) [3)]. On a regular basis the farmers are neglecting protocols for safe use of pesticides but they also continuously apply too high concentrations of pesticides [11)] which could have serious implications not only for their own health [125)] but also for the health of the consumers [133)], live stocks [134)] and the environment [135)]. In addition, as they often apply single brands of synthetic pesticides there is the risk of building up resistance in the pest against the pesticide. For all these reasons, the Indonesian government is stimulating efforts to reduce the dependency on synthetic pesticides by stimulating alternative methods including the development of formulations of botanical pesticides to control pests. The use of pesticides of plant origin instead of synthetic pesticides is expected to reduce the possible adverse effects because botanical pesticides are generally less persistent [136)] allowing local specific fighting of pests. This prevents the build-up of toxic residues on food and in natural ecosystems. In addition it is expected that pests do not easily build-up resistance against botanical pesticides, as they consist of complex mixtures of active ingredients [33)].

The aim of the present study was to formulate a botanical pesticide that could be used to control the major pests of black pepper. Based on the test with topical application, and the no-choice and the dual-choice bioassays, 10 potent plant extracts were shown to be most effective against the model pest species *Tribolium castaneum* Herbst [137)]. In the present study these 10 extracts were tested in the same assays with the major pest species of black pepper, *L. piperis* in order to quantify their toxicity and repellent effect against species from the field. Based on the three most promising extracts a new botanical pesticide formulation was defined and the concentrations of the known active ingredients in the formulation were analyzed as well. Subsequently, the formulation was tested in the laboratory for its potency against 6 pest species of black pepper i.e. *L. piperis, D. piperis*, *D. hewetti*, *Ferrisia virgata* Cockerell, *Aphis gossypii* Glover, and *A. craccivora* Koch, followed by green house and field experiments. In an additional bioassay the toxicity of the botanical formulation against the aquatic model species, *Culex pipiens* L was determined to get an indication of the toxicity of the formulation to non-target aquatic organisms.

5.2. Material and methods

5.2.1. Chemicals

DMSO (99.9% pure for spectroscopy were from Acros Organics (Geel, Belgium). Xylene (technical grade), toluene (technical grade), Tween 80 (synthesis grade), acetone (technical grade), ethanol (absolute, analysis grade) and Decis (25g of the active ingredient deltamethrin l^{-1}; CAS nr active ingredient 52918-63-5) were purchased from the agro-chemical shop, Sarana Tani in Bogor, Indonesia.

5.2.2. Plant extracts

The ten plant extracts used for the current experiments were selected out of 17 plant extracts based on their effectiveness in killing the model insect species *Tribolium castaneum* Herbst [137)]. The ten selected extracts were pyrethrum (*Chrysanthemum cinerarieaefolium* L.), sweet flag (*Acorus calamus* L.), lemongrass (*Cymbopogon citratus* L.), cashew (*Anacardium occidentale* L), clove (*Syzygium aromaticum* L.), graviola (*Annona muricata* L), tobacco (*Nicotiana tabacum* L), patchouli (*Pogostemon cablin* Benth.), neem (*Azadirachta indica* L.), and vetiver (*Andropogon zizanioides* L.). The ethanol-based extraction procedures were based on the method described by [83)]. In short 1kg material was dried in the sun for 4-5 days then grinded in a hammer mill (Reisch Mühle made by Karl Kolb (Dreieich, Germany)) using 3 mm grinders. To the 1 kg powder 5 l of ethanol (96%) was added followed by 3 hours mixing at 500 rpm using an electric mixer made by Karl Kolb (Dreieich, Germany). Subsequently, the mixture was left standing overnight in the dark at 28 ± 1ºC to allow further extraction of the active ingredients. After this, the mixture was filtered using Whatman no 91 filter paper and the residues were soaked and shaken again in 1 liter of ethanol for 2 hours. After that the solution was filtered again over a new filter and the first and second filtrate were mixed and concentrated using a rotavapor at 45°C for approximately 3 hours until all ethanol was removed and only oils were left. The extracts were transferred into brown glass bottles and stored at -4°C. In the next week about 10 ml of each extract was poured into 20 ml glass bottles and transferred to the laboratory of the Section of Toxicology of Wageningen University and stored at -20°C until further use.

5.2.3. Insects

All insects were reared in the laboratory or green houses at 29 ± 2°C, relative humidity of 65-73% and maintained at a light:dark regime of 12:12 hours. *L. piperis* was reared in the Research Location of the Indonesian Spice and Industrial Crops Research Institute (ISICRI) in Sukamulya. *L. piperis* adults were reared in glass beakers of 15 cm diameter x 25 cm height and fed with pepper plant stems of 15 cm. After a week the stems containing eggs of *L. piperis* were collected and put in other containers. The newly emerged adults of 3-4 weeks old and an average weight of 4.4 ± 0.3 mg were used for the experiments.

Dasynus piperis was reared in the Research Location of the ISICRI in Lampung. Five bushy pepper plants of 12 months old, which were growing in a generative period, were each put in a cage of 40 cm diameter x 70 cm height and used to rear 2 males and 8 female adults. Eggs laid by these females were kept in the cage and allowed to develop into adults. These newly emerged adults of 3-5 days old and an average weight of 18.3 ± 0.4 mg were used for the experiments.

Diconocoris hewetti beetles weighing 1.1 ± 0.1 mg were collected from Bangka Island and reared in the laboratory of the Indonesian Medicinal and Aromatically Crops Research Institute (IMACRI) using the same method as that of *D. piperis*. Three minor insect pest species of black pepper, *Ferrisia virgata, Aphis gossypii* and *A. craccivora* also were reared in the IMACRI. *F. virgata* was maintained on pepper plants grown in polybags and *A. gossypii* and *A. craccivora* were reared on *Gliricidia maculata* Kunth, a climbing vine of black pepper. As aquatic model insect species, larvae of mosquito of *C. pipiens* were collected from ditches on Mekarwangi village, Bogor. The third-instar stage of the mosquito larvae was used for the experiment.

5.2.4. Bioassays with plant extracts

5.2.4.1. Topical application

The direct contact toxicity was tested by topical application of 1.5 µl of the plant extracts or deltamethrin with a micropipette on the thorax of adults of *L. piperis.* The initial concentrations tested were 400 µg extract ml^{-1} solvent. Of the most toxic extracts 4 additional 2-fold dilutions were made and tested to make a full dose-response curve. In total 5 concentrations were tested including a DMSO solvent control. Meanwhile, Decis was tested in the range of its recommended dose [49)], namely 0, 0.16, 0.31, 0.63, and 1.25µg technical mixture ml^{-1} solvent resulting in a final concentrations of 0, 4, 8, 16, and 32 µg deltamethrin ml^{-1} solvent, respectively. Exposure of groups of ten adults was performed in triplicate. They were kept in 15 cm diameter x 2 cm height Petri dishes containing black pepper plant stems of 5 cm length. The Petri dishes were kept in a dark room at 29 ± 2ºC, 65-73% humidity. Mortality was recorded at 1, 3, and 5 days after application (daa). The main active ingredient of the most potent plant extract was analysed and the LC_{50} was expressed as µg active ingredient per mg of insect.

5.2.4.2. No choice bioassay

The no choice bioassay was performed according to a method described before [126]. A spike was soaked for about three seconds in 10 µl ml^{-1} of a solution of the plant extract, the synthetic pesticide deltamethrin in 1 µl ml^{-1} or solvent only (control). The spike was left to dry for 15 minutes after which it was placed in 15cm diameter x 2 cm height Petri dish. After that 10 adults of *L. piperis* were transferred to the Petri dish which was performed in triplicate. As worst case condition, a group of insects kept without any food. The mortality of the insects was recorded daily and dead insects were removed until all insects in the positive control were dead. This end-point mortality was reached on the 5^{th} day after application.

5.2.4.3. Dual choice bioassay

The repellence of the extracts against *L. piperis* was tested with stems of 5 cm long that were soaked for about two seconds in a solution of 10 µl ml^{-1} of a botanical extract or deltamethrin in 1 µl ml^{-1} and then left to dry for 15 minutes. One spike treated with an extract and another one treated with solvent only (control) were offered about 13 cm from each other in a 15 cm diameter x 2 cm height glass Petri dish. The repellence bioassay was performed in triplicate using 10 adults of *L. piperis* per dish, released in the middle of the dish. The numbers of insects present at the treated or control stem were counted at 1 and 24 hours after application (haa). The repellence index was calculated using the formula described previously [126]; R.I. = [(C-T)/(C+T)] x100%, which C being the number of insects on the control diet, and T being number of insects on the treated diet. Positive values express repellency and negative values express attractancy.

5.2.5. Formulation of the botanical pesticide and analysis of active ingredients of the selected extracts

The formulation of a botanical pesticide from the selected plant extracts was performed according to a method described before [124] with modification related to the composition of both the selected extracts and solvents. The formulation was prepared as follows: 15 ml of pyrethrum, 12 ml of sweet flag and 5 ml of clove extracts were diluted in 50 ml of a mixture of toluene and xylene. This dilution was then homogenized on an Ika KS260 basic shaker (Staufen, Germany) at 350 rpm for 2 hours. To this homogenized dilution then 8 ml of sesame oil (*Sesamum indicum* L.) was added as synergist to enhance the efficacy of the pesticide [138] and 10 ml of Tween 80 as surfactant. The solution was then re-homogenized as described above. After that, the known main active ingredients of the three extracts in the final formulation were analyzed. Pyrethrin, the active ingredient of pyrethrum [72], was analyzed according to the method described before [139], and ß-asarone and eugenol, the active ingredients of respectively sweet flag [16] and clove [27], were analyzed according to the method described by Masada [140]. The resulting product was then ready to be used for the laboratory bioassays, the green house and the field experiment.

5.2.6. Laboratory bioassays with the botanical pesticide formulation

With the newly prepared formulation the same 3 bioassays as described above i.e. topical application, no choice and dual choice bioassays were performed using three major pests of black pepper i.e. *L. piperis, D, piperis* and *D. hewetti.* The first assay with topical application was conducted to determine the concentration which was able to kill about 95% of the treated insects. Based on this finding, the formulation then also was tested in triplicate in 4 further 2-fold dilutions and a control to determine the LC_{50} and LC_{90} values in the topical application assay. The dual choice and the no choice bioassay were performed in triplicate with concentrations of 1 and 10 µl formulation ml^{-1} solvent.

In addition a bioassay was performed with three minor pest species of black pepper: *A. craccivora, A. gossypii*, and *F. virgata,* based on the leaf dipping bioassay as described before [141, 142]. In short, a leaf with the insects firmly attached on its surface was dipped in the pesticide solution for 2 seconds after which the pesticide was allowed to air dry for 15 minutes and transferred into a Petri dish of 15 cm diameter x 2 cm height. The tested concentrations were 0, 0.0001, 0.001, 0.01, 0.1, 1, and 10 µl ml^{-1} water and the exposures were performed in triplicate. About 1 and 24 hours after application (haa) the number of dead and living insects were counted and the LC_{50} and LC_{90} values were calculated. For these tiny insects, the LC $_{50}$ and LC_{90} values were expressed in µl ml^{-1} dipping water.

With the newly defined botanical formulation a bioassay with the aquatic larvae of *C. pipiens* was conducted based on the method described before [143]. In short, triplicate groups of 20 larvae were placed in glass bottles of 10 cm diameter x 15 height containing 100 ml of fresh tap water with the formulation in the concentrations 0, 0.03, 0.05, 0.08, 0.1, 0.13, and 0.15µl ml^{-1} water. Twenty four hours after application the dead and living larvae were counted. The criterion of death was a lack of physical response upon tapping on the bottle.

5.2.7. Green house experiment with the botanical pesticide formula

Based on concentrations determined in the laboratory bioassays, toxicity of the formulation was tested in green house experiments with *L. piperis, D. piperis*, and *D. hewetti.* The insects were reared in bushy pepper plants of 12 months old, which were growing in a generative phase. Each plant was put in a cage of 40 x 40 x 70 cm^3 and used to rear 10 adults of each pest species and left to stand for 2 days to adapt to the environmental condition. The experiment was conducted in triplicate. For each experiment about 10ml of botanical pesticide solution was sprayed evenly onto the pepper plant using a 1 l hand sprayer (Canyon purchased from the Sarana Tani, Bogor, Indonesia). The applied concentration, chosen based on the results of the laboratory experiments, was 10 µl ml^{-1} water. Mortality of the pest species was determined 1, 24, 48 and 96 hours after application (haa).

5.2.8. Field experiment with the botanical pesticide formulation

Finally the newly defined botanical pesticide formulation was tested twice in a field experiment performed at the Lampung Experimental Garden of the ISICRI. Both the botanical pesticide formulation and the recommended synthetic pesticide deltamethrin were sprayed onto the pepper plants according to the common habits of local farmers as described before [11)]. The concentration of the botanical formulation was based on the results of the laboratory and green house experiments, while that of the synthetic pesticide was based on its recommended concentration. Each pesticide was applied evenly onto pepper plants. Before commencing the application, a piece of $1m^2$ white cloth was spread out around the stem of a pepper plant to catch the falling or dead organisms after pesticide application (Figure 7.). The number of dead organisms collected from 5 sprayed plants was counted 1, 3, 9, 27 and 81 hours after application (haa). The experiment was repeated in the same experimental garden.

Figure 7. Pepper plants used for the field experiment. Two branches were covered with two nylon bags which were use to rear *L. piperis* or *D. hewetti* before commencing field experiments

To evaluate the effectiveness of the botanical formulation in more detail the major pests of black pepper in the study area, *L. piperis* and *D. piperis* were reared on the pepper plant branches which would be treated as well during the field application according to the method described before [8)]. In short, two branches of a pepper plant containing young berries of about 2 months old were covered with two nylon bags (Figure 7) of 30 cm diameter x 50cm height with 1x1mm^2 pores. Each bag had a zip of 20 cm long, via which the insects to test were added. The first bag was filled with 10 adults of *L. piperis* while the second bag was filled with 10 adults of *D. piperis*. Before commencing with the pesticide application, the insects were allowed to adapt to the field conditions for 2 days. This procedure was applied to 5 plants for each pesticide which were sprayed with either the botanical formulation or the recommended synthetic pesticide as described above. The mortality of the pest insects was observed at 1, 3, 9, 27 and 81 haa..

5.2.9. Data analysis

The number of dead insects was corrected for the mortality in the controls using Abbott's formula [85)]. The lethal concentrations, LC_{50} and LC_{90} values were determined using the program Slide Write Plus 6.1 (Advanced Graphics Software Inc.). Statistical significance between the treatments was determined with Duncan's multiple range test (DMRT) with 95% confidence interval.

5.3. Results

5.3.1. Bioassays with the plant extracts against L piperis

After topical application only the extract of pyrethrum was able to kill all *L. piperis* when using 1.5 µl of 400 µg extract ml^{-1} solvent. With the extracts of clove, sweet flag, lemongrass, patchouli, and cashew only about 3% of the insects were killed and the other plant extracts were not effective at all (data not shown). In the pyrethrum extract the concentration of pyrethrin, the main active ingredient, was 12.4%.

In further tests with lower concentrations of the pyrethrum extract the LC_{50} and LC_{90} at 48 haa were determined to be 6.8 and 30.8 µg extract ml^{-1} solvent, respectively, equivalent to 0.19 and 0.87 µg pyrethrin mg^{-1} insect. For the synthetic pesticide Decis (25 g deltamethrin l^{-1}) the LC_{50} and LC_{90} were 0.31 µg technical mixture ml^{-1} solvent equivalent to 1.76µg deltamethrin mg^{-1} insect (Table 14). Thus the toxicity of the natural pyrethrin in the pyrethrum extract at 48 haa was 2 times lower than that of the synthetic pyrethroid deltamethrin.

In the no choice bioassay extracts from pyrethrum and sweet flag were able to kill 21 and 7% of the insects, respectively. Other extracts tested had no effect at all. Meanwhile, in the dual choice bioassay, several extracts were able to repel *L. piperis.* The highest repellency index was found for the extract of pyrethrum (87%) followed by sweet flag (73%), clove (70%), and lemongrass and cashew (67%) (Table 15).

Table 14. Corrected mortality of *L. piperis* treated by pyrethrum extract and Decis on 24 and 48 hours after application (haa). LC_{50} and LC_{90} were calculated for 48 haa expressed as µg active ingredient mg^{-1} insect.

Extract/ pesticide	Concentration (µg ml-1)	Average of mortality (% ± SE)		48 haa	
		24 haa	48 haa	LC_{50}	LC_{90}
Pyrethrum	0.0	0 ± 0.0 c	0.0 ± 0.0 c		
	6.3	70 ± 16.7 b	53.3 ± 8.8 b		
	12.5	87 ± 3.3 b	60.0 ± 10.0 b	0.03	0.14
	25.0	87 ± 6.7 a	86.7 ± 6.7 a		
	50.0	100 ± 0.0 a	100.0 ± 0.0 a		
Decis	0.0	0.0 ± 0.0 d	0.0 ± 0.0 d		
	0.155	23.3 ±. 3.3 c	23.3 ±. 3.3 c		
	0.313	50.0 ± 5.8 b	50.0 ± 5.8 b	1.76	1.76
	0.625	93.3 ± 6.7 a	93.3 ± 6.7 a		
	1.25	100.0 ± 0.0 a	100.0 ± 0.0 a		

Means in the same column for each compound with the same superscripted letters are not significantly different (P>0.05).

Table 15. Average mortality and repellency index of *L. piperis* treated by plant extracts.

Name of plants	Average ± SD	
	No choice bioassay Mortality (%)	Dual choice bioassay Repellency index (%)
Pyrethrum	20.8 ± 12.1	86.7 ± 6.7 [a]
Sweet flag	6.7 ± 6.7	73.3 ± 6.7 [a]
Clove	0	70.0 ± 1.5 [a]
Lemongrass	0	66.7 ± 6.7 [a]
Cashew	0	66.7 ± 6.7 [a]
Graviola	0	60.0 ± 1.5 [a]
Tobacco	0	53.3 ± 6.7 [a]
Patchouli	0	53.3 ± 6.7 [a]
Neem	0	53.3 ± 6.7 [a]
Vetiver	0	53.3 ± 6.7 [a]
Deltamethrin	100 ± 0	53.3 ± 6.7 [a]

Means in the same column for each compound with the same superscripted letters are not significantly different (P>0.05).

Based on these findings, extracts of pyrethrum, sweet flag and clove containing respectively pyrethrin, ß-asarone, and eugenol as their active ingredients, were selected to define the new botanical formulation. Pyrethrum was effective in topical, no choice and dual choice bioassays, while sweet flag and clove were effective in dual choice bioassay. Results of chemical analysis showed that the formulated botanical pesticide contained 1.9% pyrethrin, 7% ß-asarone, and 4% eugenol.

5.3.2. Bioassays with the botanical pesticide formulation against pests of black pepper.

Topical application of the newly defined botanical pesticide formulation revealed that 900, 75 and 20 µl ml^{-1} of the formulation killed about 95% of *L. piperis, D. hewetti* and *D. piperis*, respectively. Based on these findings the formulation was further tested in the range of 0, 60, 125, 250, 500µl ml^{-1} water against L. piperis in a range of 0, 8, 17, 35, and 70µl ml^{-1} against *D. hewetti*, and in a range of 0, 2, 4, 8, 16µl ml^{-1} against *D. piperis*. The lowest LC_{50} and LC_{90} were found for D. piperis followed by *D. hewetti* and *L. piperis* (Table 16).

Table 16. Lethal concentration of botanical formulation against major pests of black pepper and mortality and repellency index of the formulation treated on 10µl ml^{-1} water. Experiments were conducted in triplicate with 10 insects per replication.

Tested insects	Topical application		No choice bioassay			Dual choice bioassay	
	Lethal Concentration (µg pyrethrin mg^{-1} insect)		Mortality (%)			Repellency Index (%)	
	LC_{50}	LC_{90}	2daa	4daa	6daa	1hr	24hrs
L. piperis	1.72	2.41	10	34	46	96	80
D. piperis	0.05	0.02	0	0	0	35	14
D. hewetti	0.60	1.170	0	0	0	60	20

In the no-choice bioassay 1µl formulation ml^{-1} water did not kill the treated insects, while that of 10µl ml^{-1} water was able to kill 10% of *L. piperis*. This mortality increased in time to 34 and 46% on 4 and 6 daa, respectively. Meanwhile, no mortality was found for *D. piperis* and *D. hewetti* with either of the two concentrations.

The dual choice bioassay showed that at the concentration of 10 µl ml^{-1} the formula slightly repelled the treated insects. The repellency index (RI) for *L. piperis* was 96% at 1 haa which decreased to 80% on the following day (24haa). Meanwhile the RI of the botanical formulation when tested in the same concentration against *D. piperis* was 35% which decreased to 14% on 24 haa. The RI of the formulation against *D. hewetti* was 60% decreasing to 20% on 24 haa.

5.3.3. Effectiveness of the newly defined botanical pesticide formulation in bioassays with three minor insect pests of black pepper and mosquito larvae

The bioassays with the formulation tested against the three minor pests of black pepper i.e. *A. craccivora*, A. gossypii, and *F. virgata* showed that the formulation was effective against these pests as well. The LC_{50} and LC_{90} of the formulation against *A. craccivora* at 24 haa were as low as 0.03 and 0.8 µl ml^{-1} followed by those of *A. gossypii* (4.6 and 8.9 µl ml^{-1}) and of *F. virgata* (5.4 and 9.7 µl ml^{-1}) (Table 17). The larvae of the mosquito, C. pipiens were very sensitive to the formulation, and at 24 haa the LC_{50} and LC_{90} of the formulation in their water were 0.04 and 0.14 µl ml^{-1}, respectively.

Table 17. Average toxicity of the botanical pesticide formulation against *A. craccivora, A. gossypii*, and *F. virgata* on 24 haa in the leaf dipping bioassay conducted in triplicate. LC_{50} and LC_{90} were expressed in µg extract ml^{-1} water.

Concentration (µl ml^{-1})	% Mortality (mean ± S.D.) on 24 haa		
	A. craccivora	*A. gossypii*	*F. virgata*
0	0.0 d	0.0 d	0.0 b
0.001	20.0 ± 2.9 c	0.0 ± 0.0 d	0.0 ± 0.0 b
0.01	86.7 ± 1.7 b	8.3 ± 1.7 c	0.0 ± 0.0 b
0.1	100 a	18.3 ± 1.7 b	0.0 ± 0.0 b
1.0	100 a	21.7 ± 3.3 b	8.3 ± 1.7 b
10	100 a	100 a	93.0 ± 3.6 a
LC50 (µl ml-1)	0.03	4.57	5.40
LC90 (µl ml-1)	0.8	8.86	9.69

Means in the same column for each compound with the same superscripted letters are not significantly different (P>0.05).

5.3.4. Green house experiment with the botanical pesticide formulation

The green house experiment was conducted to evaluate the toxicity of 10 µl formulation ml^{-1} water, a concentration that was considered most suitable based on the laboratory and greenhouse experiments. In the laboratory experiments this concentration was able to kill 92% of *D. piperis* and 75% of *D. hewetti*. In the green house experiment the same concentration was able to kill 87% of *D. piperis* and 60% of *D. hewetti* (data not shown) but was not effective against *L. piperis*.

5.3.5. Field experiment with the botanical pesticide formulation

Because of the extreme rainfall on Bangka Island during the planned period for the experiments, the field experiments were conducted in the Lampung province. In this province only two major pests are found on the pepper plants i.e. *L. piperis* and *D. piperis*. The results of the field experiments showed that the botanical formulation was effective against most pests living on the treated plants and relatively safer for natural enemies of pest species. The time needed for recolonization of the pepper plants treated with the botanical pesticide was shorter than the time needed after applying the synthetic pesticide.

The field experiment showed that at 27 haa the botanical formulation was able to kill 60% of *D. piperis* and 0% of *L. piperis* living in the cages. Observation of the number of insects falling on the cloth underneath the plant showed that the botanical formulation was able to kill, in addition to the green pepper bug, D. piperis, lady birds, queen and weaver ants, jumping and lynx spiders, egg parasitoids, rice and shorthored grasshoppers, and cockroach but was not killing *L. piperis, Batocera* sp, and *S. lurida*. Fortunately, the botanical formulation did not kill S. piperis, a natural enemy of *L. piperis*, a key pest of the black pepper, *Sycanus* sp. and *Otomantis* sp, natural enemies of many insect pest species, as well as *M. domestica*, and *Telegryllus* sp., common insect species frequently found in the environment. The recommended synthetic pesticide, Decis (25 g deltamethrin/l), killed all organisms living in the cages and on the treated plants (Table 18).

At nine haa 90% of the plants treated by the botanical pesticide formulation were recolonized again by two pest species i.e. weaver ants, *Oecophylla smaragdina* (F.) and lynx spiders, *Oxyopes* sp. Meanwhile at that same time point only 20% of the plants treated by Decis were recolonized again by the ants. These ants, however, died shortly after climbing the synthetic pesticide-treated plants. At 71 haa the weaver ants still died on the plants treated by the synthetic pesticide, while there was no mortality on the plants treated by the botanical pesticide formulation. Independent repetition of the field experiment yielded similar results as the first experiment. The pesticide was not effective against *L. piperis*, but was able to kill 70% of the green pepper bug, *D. piperis* exposed in the cages. The botanical formulation also was effective against other pests associated with the treated plant such as *Acrida Turrita* (L.), and *Valanga nigricornis* (Burm.). Fortunately, the botanical pesticide was safe for the 3 species of natural enemies of the black pepper insect pests found, namely *Iridomyrmes* sp., *Gryion dasyni*, and *Anastatus dasyni* (Ferr.). Meanwhile, the synthetic pesticide killed 100% of the *D. piperis* exposed in the cages and all organisms living on the treated plants (Table 19).

5.4. Discussion

Our current study with plant extracts showed that extracts of pyrethrum, sweet flag, and clove were the most potent extracts to be included when developing a botanical pesticide formulation. Application of the formulation containing these extracts gave promising results as the formulation was effective against pests of black pepper and relatively less toxic against natural enemies of the pests.

These results are in accordance with other studies in the literature reporting on the toxicity of these plants towards various pest species. Pyrethrum extract has been reported before to be effective against *Sitophilus granarius* (L) [17], *Rhyzopherta dominica* (F) [26] and *Tribolium confusum* (DuVal) [15]. Sweet flag extract was shown to be effective to control *Prostephanus truncatus* (Horn) [16], *Lasioderma serricorne* F, [115], *Sithopylus oryzae* (L), *Callosobruchus chinensis* (L) [116] and *C. phaseoli* (Gyllenhall) [18]. The pest species that clove extract has been reported to be effective against are *T. castaneum* (Herbst) and *S.* zeamais (Motsch) [27], *Dermanyssus gallinae* (De Geer) [19] and *Iodes ricinus* (L) [20].

This study not only presents the toxicity of the extracts against several pest species of black pepper, it also reveals that the application of the botanical formulation provides an effective and environmentally safer method to control pests of black pepper in plantations. The formulation contained sesame oil, xylene, toluene, and Tween 80 to enhance the bioavailability of the extracts and proved to be effective against the notorious pepper pests *D. piperis* and *D. hewetti* that currently reduce the pepper production with about 15% [144] and 39% [145], respectively. The formula also was effective against three minor pests of black pepper, *A. gossypii, A. craccivora*, and *F. virgata* and two grasshopper species, *Acrida Turrita* and *Valanga nigricornis.*. Based on these findings, and because of the similarities in the biology of the species, we expect that the formulation could control some minor pests from the order of i.e. orthoptera, lepidoptera, gastropoda, homoptera, hemiptera, and hymenoptera at the irregular occasions when their populations suddenly increase.

The field experiment revealed that the formulation was more selective in killing pest species than the synthetic one as 3 species of natural enemies i.e. *Dolichoderus* sp., *Otomantis* sp, and *Sycanus* sp. survived following the application of the botanical formulation but were killed by the synthetic one. The selectivity of a pesticide is crucial in pest management strategies since natural enemies play an important role in the fight against a pest and killing of the natural enemies could result in a secondary pest. When natural enemies are able to control pest populations to remain under the economic threshold they have the potential to mitigate pest control cost [146] by reducing the number of pesticide applications.

Against the aquatic organisms *C. pipiens* the LC_{50} of the botanical formulation was 0.04 µl ml^{-1} indicating that for this aquatic non-pest species the formulation is about 2000 times less toxic than Decis with an LC_{50} of 0.00002µl technical mixture ml^{-1} [147]. Compared to another recommended synthetic pesticide used in pepper plantations, Dursban[49] with a reported LC_{50} of 0.0005 µl ml^{-1} [148], the formulation was about 80 times less toxic.

This indicates that the formulation is not as toxic as the synthetic pesticide against this mosquito larva and possibly against other natural aquatic organisms. It may, however, still be interesting to see whether this formulation could be of use in case of the anticipated further development of mosquitoes-vector diseases such as Dengue Haemmoraghic Fever,

Table 18. Number of dead animals after application of botanical and synthetic pesticides on pepper plantation. Information includes status, order, scientific names and common names of the animals in the first field experiment.

Status	Order	Scientific names	Common names	Deltamethrin Hour(s) 1	3	9	27	Total	Botanical pesticide Hour(s) 1	3	9	27	Total
Pests													
	Coleoptera	*L. piperis*	Stem borer	43	14	4	17	78	0	0	0	0	0
	Hemiptera	*D. piperis*	Green pepper bug	0	0	0	0	0	1	0	0	0	1
	Coleoptera	*Batocera sp*	Stem borer	1	0	0	0	1	0	0	0	0	0
	Hemiptera	*S. lurida*	Black rice bug	10	9	0	0	19	0	0	0	0	0
Natural enemies													
	Coleoptera	*C. transversalis*	Lady birds	3	3	0	0	6	5	0	0	0	5
	Heteroptera	*Sycanus sp*	Assassin bugs	5	3	0	2	10	0	0	0	0	0
	Mantodea	*Otomantis sp*	Praying mantis	0	1	0	0	1	0	0	0	0	0
	Hymenoptera	*Iridomyrmex sp.*	Queen ant	0	0	0	0	0	5	0	0	0	5
	Hymenoptera	*Anastatus dasyni*	Egg parasitoid	1	2	0	0	3	1	0	0	0	1
	Hymenoptera	*Spathius piperis*	Larvae parasitoid	2	1	0	0	3	0	0	0	0	0
	Hemiptera	*O. smaragdina*	Weaver ant	1,600	470	640	10	2,720	1,500	40	0	0	1,540
	Hemiptera	*Gryon dasyni*	Egg parasitoid	0	0	0	0	0	1	1	0	0	2
	Aranae	*Lycosa sp*	Jumping spider	17	14	0	1	32	5	1	0	0	6
	Aranae	*Oxyopes sp.*	lynx spider	11	9	0	0	20	2	0	0	0	2
Others													
	Blattodea	*P. americana*	Cockroach	3	0	0	0	3	1	0	0	0	1
	Diptera	*M. domestica*	Housefly	1	1	0	0	2	0	0	0	0	0
	Orthoptera	*Teleogryllus sp*	Cricket	2	1	0	0	3	0	0	0	0	0

Table 19. Number of dead animals after application of botanical and synthetic pesticides on pepper plants. Information includes status, order, scientific names and common names of the animals in the second field experiment.

Status	Order	Scientific names	Common names	Deltamethrin Hour(s)				Total	Botanical pesticide Hour(s)				Total
				1	3	9	27		1	3	9	27	
Pests													
	Coleoptera	*L. piperis*	Stem borer	1	0	0	2	3	0	0	0	0	0
	Hemiptera	*D. piperis*	Green pepper bug	4	1	0	0	5	2	0	0	0	2
	Orthoptera	*A. Turrita*	Rice grasshopper	5	0	0	2	7	4	0	0	0	4
	Orthoptera	*V. nigricornis*	Short horned grasshopper	2	0	0	0	2	3	1	0	0	4
Natural enemies													
	Coleoptera	*C. transversalis*	Lady birds	1	0	0	0	1	2	0	0	0	2
	Heteroptera	*Sycanus* sp	Assassin bugs	6	2	0	3	11	0	0	0	0	0
	Mantodea	*Otomantis* sp	Praying mantis	1	0	0	0	1	0	0	0	0	0
	Hymenoptera	*Iridomyrmex* sp.	Queen ant	6	1	0	0	7	0	0	0	0	0
	Hymenoptera	*A. dasyni*	Egg parasitoid	1	0	0	0	1	0	0	0	0	0
	Hymenoptera	*Spathius piperis*	Larvae parasitoid	0	0	0	0	0	0	1	0	0	0
	Hemiptera	*O. smaragdina*	Weaver ant	300	2	125	0	427	27	4	0	0	31
	Hemiptera	*Gryon dasyni*	Egg parasitoid	2	0	0	0	2	0	0	0	0	0
	Aranae	*Lycosa* sp	Jumping spider	4	2	0	0	6	3	0	0	0	3
	Aranae	Oxyopes sp.	lynx spider	2	1	1	0	4	4	2	0	0	6
	Hymenoptera	*Dolichoderus* sp.	Black ant	22	1	0	0	23	0	0	0	0	0
Others													
	Blattodea	*P. americana*	Cockroach	4	1	0	1	6	9	1	0	0	10
	Diptera	*M. domestica*	Housefly	1	0	0	0	1	0	0	0	0	0
	Orthoptera	*Teleogryllus* sp	Cricket	5	2	0	1	8	4	0	0	0	4

Malaria, and Filariasis caused by *Aedes aegypti*, *Anopheles aconitus*, and *C. quinguefasciatus*, respectively, because pest resistance is not expected to occur easily since botanical pesticides consist of complex mixtures of active ingredients [33]. This in contrast to the synthetic pesticide deltamethrin, for which building up of resistance of *C. pipiens* Pallens was shown following constant application for 12 generation, with the LC_{50} value rising steadily from 0.040 mg/l to 24.660 mg/l[149].

The formulation is expected to have the additional advantages of suppressing the development of root rot and yellowing diseases of pepper as it contains eugenol as one of its active ingredients, which has been reported to act as a potent fungicide [150] and nematicide [14, 151]. More specifically eugenol has been reported effective against the fungus *Phytophthora palmivora* causing root rot disease [23] and the nematode *Meloidogyne incognita* causing yellowing disease [152]. Although in this study the safety of our formulation for the farmers or the consumers was not tested yet, the safety for the environment seems to be much greater as at 9 haa the treated plants already were recolonized again by ants. Meanwhile, at 71 haa the ants still died after climbing the synthetic pesticide treated plants. This is in accordance with the reported faster degradation of botanical pesticides compared to that of synthetic pesticides [72]. This finding is important because farmers really depend on the use of pesticides and currently most of them use synthetic pesticide unwisely leading to health risks for the consumer and environment [11]. A safety evaluation of the consumption of black pepper berries treated with botanical pesticides revealed that no consumer health risks are to be expected [153].

Overall, we conclude that the use of our botanical pesticide formulation based on extracts from pyrethrum, sweet flag, and clove could offer a promising strategy to control *D. piperis* and *D. hewetti* and a number of minor pests in black pepper plantations. The botanical formulation had lower toxicity against several natural enemies of the pest species organisms and had a shorter degradation period compared to that of synthetic pesticides. The greater biodegradability also reduces the risk for consumers of berries treated with the formulation compared to berries treated with recommended synthetic pesticides such as Decis. As the ingredients are locally available and the formulations does not require expensive ingredients, it could be produced locally thus reducing the dependency on imported synthetic pesticides.

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Chapter

6

Consumer Safety Evaluation of Application of Botanical Pesticides in Black Pepper Crop Protection

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Abstract

Aim of the study; to perform a consumer safety evaluation of the application of four botanical pesticides used in pepper berry crop protection, including preparations from *Syzygium aromaticum* (Clove), *Derris elliptica* (Tuba root), *Acorus calamus* (Sweet flag), and *Chrysanthemum cinerarieaefolium* (Pyrethrum). Materials and Methods; Based on i) oral toxicity data from human and animal studies with these botanical pesticides or their main active ingredients and ii) estimated exposure resulting from use of these extracts in pepper berry crop protection, a safety evaluation was performed. Results; Botanical pesticides derived from *A. calamus* appeared to be of possible concern because of the genotoxic and carcinogenic ingredient beta-asarone. However, based on beta-asarone cancer data and a worst case intake estimate resulting from the proposed use an Margin of Exposure (MoE) was determined that was higher than 4 $x10^6$. For the other botanical pesticides evaluated a margin of safety could be determined which was at least 500. Conclusions; Use of extracts of *A. calamus* containing beta-asarone would result in a MoE that would not indicate a high priority for risk management. However, because for beta-asarone restrictions in applications as food additive are indicated and since use as a pesticide implies an avoidable risk, it is concluded that the application of this botanical pesticide should not be encouraged. The use and use levels of the other three botanical pesticides evaluated are not of safety concern.

Key words: Botanical pesticides, clove, pyrethrum, safety evaluation, sweet flag, tuba root

6.1. Introduction

At present farmers in Indonesia mostly use synthetic pesticides to manage pests in pepper plantations, posing potential risks for local workers, consumers and the environment [11]. Concerns over health and environmental problems associated with the synthetic pesticides currently in use in agriculture has led to an intensification of efforts to find safe, effective, and viable alternatives. Hence, in recent years efforts have been directed towards studying active ingredients derived from natural products to develop plant-based botanical pesticides. Botanical pesticides may answer the need for safer compounds to protect plantations from attack by a wide range of pests [113].

In the search for environmentally friendly pesticides, much research has been done on the use of plants for the protection of crops in the field or in storage. Especially in tropical regions, the application of botanical material to protect a crop against pests is often traditional and centuries old. However, if botanical pesticides are to be used for the protection of crops in the field and/or to treat stored products against insects, the health of people applying the formulation and of those consuming the crops with possible residues should not be affected.

In a series of experimental studies the potential and effectiveness of extracts of botanical origin were investigated in some detail in our laboratory. A selected series of 17 botanical and some relevant synthetic pesticides were tested in *in vivo* assays, as well as in field studies and field experiments [154].The *in vivo* assays were performed using the root-knot nematode, *Meloidogyne incognita*, the model insect *Tribolium castaneum*, and pest species of black pepper including *Lophobaris piperis*, *Ferrisia virgata*, *Aphis gossypii*, *A. craccivora* and *Culex pipiens*. The initial results of these studies indicated that especially four botanical extracts may provide effective alternatives to control pest populations. These selected botanical pesticides include extracts from *Syzygium aromaticum* (Clove), *Acorus calamus* (Sweet flag) and *Chrysanthemum cinerarieaefolium* (Pyrethrum). A recent field study investigating the farmers' awareness, and uses of synthetic and botanical pesticides by local workers in black pepper plantations on Bangka Island, Indonesia, indicated that Tuba root extract is being used as a botanical pesticide by local farmers [154], therefore this plant and its extract are also included in this review.

Given the potential use of extracts derived from the plants as pesticides and the realistic chances of residues derived from the treatments still being present at the time of consumption, one should also examine what could be the potential risk for farmers and consumers. Therefore, in this study we reviewed the toxicological data from human and animal studies with oral exposure to preparations from these four potential botanical pesticides or their known main active ingredient. Based on the overview obtained and the estimated exposure resulting from the use of these extracts as botanical pesticides a safety evaluation is performed.

Although the part of the plant extracted for use as a pesticide is well defined, data on toxicity of the botanical preparations can refer to very different preparations. These can be oil derived from crude plant parts, the seeds, flowers or leaves, aqueous extracts of parts of the plant, extracts obtained with non-aqueous solvents, the purified bioactive insecticide ingredients and/or sometimes even commercially produced botanical-based pesticides.

The first three preparation methods would be best applicable for low-resource farmers in tropical countries, where no complex extractions can be performed due to expensive solvents and lack of appropriate equipments. The other preparation methods could be valuable in countries where regulations require exact definition of the ingredients of pesticides.

To get an overview of the potential toxicity of the four selected botanical species all available mammalian toxicity data were included, specifying the nature of the extract or botanical preparation whenever this information was available. It should be stressed that the present review focussed only on risk for the consumer of the pepper berries cultivated using the botanical pesticides. Evaluation of occupational risks upon inhalation, skin exposure or oral exposure upon inadequate handling of the material by farmers was not the objective of the present safety evaluation.

6.2. Clove

Clove is the dried bud (Figure 8a) of *Syzygium aromaticum* (Figure 8b) (synonyms are *Carophyllus aromaticum*, *Eugenia aromatica* or *E. caryophyllata*), a tree that is indigenous to India, Indonesia, Zanzibar, Mauritius and Ceylon [155)]. Clove is reported to be active as an aphrodisiac, nervous stimulant, tonic and Clove contains about 14-21% of volatile oil [155)]

Clove oil is an essential oil which is used in foods as an aromatic and food flavouring agent and in pharmaceutical products where clove oil and also its active component eugenol (Figure 8c) are frequently used in dental practices, as an analgesic or antiseptic agent or the home anaesthesia of tropical fish [156)]. The oil is widely available without prescription in quantities varying from 8-60 ml [156)]. This wide availability of clove oil is the basis for the documentation of various case reports of toxicity in man including children.

Other studies reporting effects of clove or clove-derived materials on mammalian systems refer to clove powder, high molecular weight polysaccharides extracted from clove flower buds, ethanol extract of clove powder, or clove oil obtained from clove buds, leaves or stem by steam distillation.

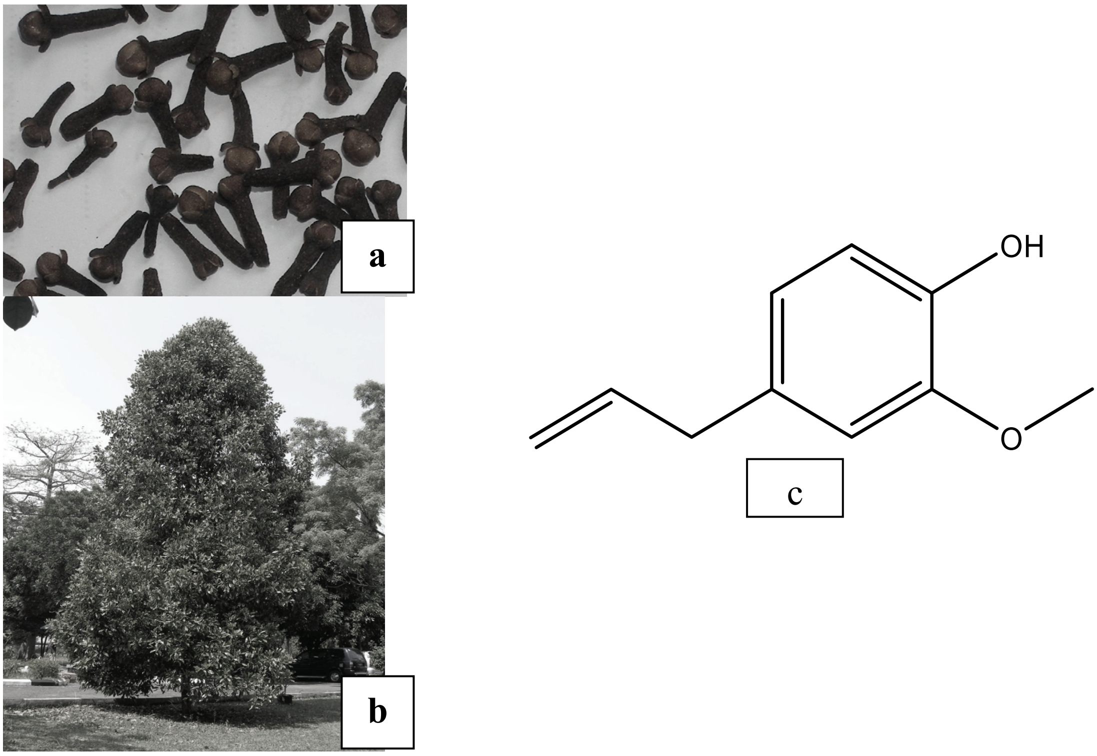

Figure 8. Dried flower buds (a), clove tree (b), and structure formula of eugenol (c).

6.2.1. Effects of Clove extracts on humans

Table 20 summarises the reported cases of toxic effects of clove-based preparations on humans following intended or accidental ingestion of clove oil.

A case study of a 3-month old 6 kg female child that was accidentally given no more than 8 ml of clove oil to drink was described [156]. The child developed fulminant hepatic failure that was successfully treated by N-acetylcysteine treatment. This suggests a mechanism of hepatic toxicity similar to acetaminophen toxicity and was ascribed to the eugenol present as a major constituent in the clove oil. Given a level of 60-80% eugenol in the essential oils from *S. aromaticum (clove)* [157], 8 ml would amount to an intake of about 4800 to 6400 mg of eugenol which, for a 6 kg child, is equivalent to 800 to 1066 mg eugenol/kg bw.

A similar case study was described in which a 2 year old boy ingested approximately 10 ml of clove oil and developed disseminated intravascular coagulation and hepatocellular necrosis [77]. Estimating the body weight of a 2 year old boy at 15 kg this implies an estimated intake of 400 to 533 mg eugenol/kg bw.

Accidental clove oil ingestion of one full teaspoon (estimated to amount to 2 ml) was described for a 7-month old child that subsequently developed central nervous system depression, urinary abnormalities and acidosis [158]. With an estimated body weight of 8 kg the estimated intake would amount to about 150 to 200 mg eugenol/kg bw which is somewhat lower than the estimated intake reported by the authors themselves of 500 mg eugenol/kg bw which was based on 5 ml intake and an estimated eugenol level of the oil amounting to even 84-88% in oil obtained from clove leaves by steam distillation [158)].

Table 20. Effects of clove *(S. aromaticum)* oil on humans (reported case studies)

Plant part	Patient	Administration	Dose	Duration	Observed effects	Reference
Clove oil/ commercial	2 years old child	oral	10 ml	single dose	Disseminated intravascular coagulation; hepatic necrosis	77)
Clove oil	3 months old 6 kg female	oral	< 8 ml	single dose	Hepatic failure and intravascular coagulation	156)
Clove oil	7 months old child	oral	One full teaspoon		Depression of central nervous system, acidosis	158)
Clove oil	Child 2 years old	oral	5-10 ml		Depression of central nervous system, acidosis, hepatic failure	159)

Finally, Hartnoll *et al.* [159)] reported a nearly fatal ingestion of oil of cloves by a 2 year old child in which 5-10 ml of clove oil ingested orally caused depression of the nervous system, acidosis and hepatic failure. Based on the same assumptions as above the intake causing these effects is estimated to have amounted to a value between 200 and 533 mg eugenol/kg bw.

Overall these case studies reveal that acute oral toxicity of clove oil is observed at doses between 150 and 1000 mg eugenol/kg bw and results in depression of the nervous system, acidosis and hepatic failure.

6.2.2. Effects of Clove extracts and eugenol on animals

The effects of clove derived materials on animals are summarised in Table 21.

6.2.3. Acute effects of clove extracts and eugenol on animals

Oral administration of 50% ethanolic extracts of powder of *S. aromaticum* to adult Swiss mice at a level of 500 mg extract /kg bw caused increased aphrodisiac activity enhancing the sexual behaviour of male mice [155)]. Although this study was not designed to detect

Table 21. Effects on animals of oral exposure to clove *(S. aromaticum)* or clove derived materials

Plant part	Test animals	Administration	Dosages	Duration	Observed effects	Reference
eugenol	mouse	oral	unknown	unknown	LD50= 3000 mg/kg bw	[160] cited in JECFA 1967
eugenol	rat	oral	unknown	unknown	LD50= 1930 mg/kg bw 2680mg/kg bw	[161, 162]
eugenol	guinea pig	oral	unknown	unknown	LD50 =2130 mg/kg bw	[160] cited in JECFA 1967
eugenol in corn oil	20 male rats	oral	1400-4000 Initial 1400 mg/ kg bw increased gradually in 34 days to 4000 mg/kg bw in the same animals	34 days	8 animals survived 34 days, 15 lived long enough to receive the maximal dose of 4000 mg/kg but had severely damaged forestomach mucosa and moderate hyperkeratosis of forestomach statisfied squamous epithelium.	[163, 164]
eugenol	10 male and 10 female rats	In the diet	0-1% in the diet (= 0-50 g/kg bw/day)	19 weeks	No effects observed.	[163]
eugenol	15 male and 15 female rats	In the diet	79.3 mg/kg bw/day	12 weeks	No effects observed	[165]
50% Ethanol extract from clove powder	Adult Swiss mice	oral	500mg extract /kg bw.	One dose	Increased aphrodisiac activity	[155]
clove powder	Male Swiss albino mice	oral	Concentration ran from0-2% (w/w) in the diet (equal to 0-2850 mg/kg bw/day)	10-30 days	NOAEL=***for biomarker responses (hepatic Cyt b5, DTD, GST and SH levels, induction, decreased CyP-450 and MDA levels	[166]
eugenol	4 dogs	Intragastric	200-500 mg/kg body weight	One dose	Dose dependent effects: 200 mg no effect 250 mg some vomitting 500 mg effect in 2 of 4 animals.	[167]

toxicity of this clove extract, the authors indicate that no conspicuous general short term toxicity was observed at the administered dose of the ethanolic clove powder extract.

An evaluation of the acute toxicity of the major constituent of clove oil, eugenol, is presented by the JECFA and the WHO in their evaluation of this food flavouring [165, 168, 169]. Acute toxicity after high doses of 250 mg eugenol/kg bw. results in vomiting in dogs and the 220mg/kg was without effect [165]and hepatoxicity in rats dosed with 900 mg eugenol//kg bw [162, 167] references from [165], but also in desquamation of the gastric mucosa [170] (reference from [168]. Punctate haemorrhages were observed in rats and guinea-pigs given 150 mg/animal orally [171] data retrieved from [165], gastric inflammation and depression of secretory capacity [168] intake not specified.

The LD_{50} of eugenol upon oral intake was reported to be 3000 mg eugenol/kg bw in mice [160], 1930 mg eugenol/kg bw in rats [161] , 2680 mg eugenol/kg bw in rats [161, 162] and 2130 mg eugenol/kg bw in guinea pigs [160],. These rodent oral LD_{50} values of about 2000-3000 mg eugenol/kg bw are only 2 to 3 times higher than the highest estimated intake in the human case studies.

6.2.4. Subchronic effects of clove extracts and eugenol on animals

Subchronic studies on the effects of clove derived material on animals is restricted to a study in which clove powder was administered to male Swiss albino mice at doses of 0, 0.5, 1 and 2% in the diet (amounting to approximately 0, 750, 1500 and 3000 mg clove powder/kg bw/day for 10, 20 and 30 days (Kumari et al. 1991). Assuming an eugenol content of clove powder of 80 % this amounts to doses of 0, 600, 1200, and 2400 mg eugenol/kg bw. At all dose levels applied the clove diet did not affect food intake or the patterns of body weight gain or liver weight gain. Various hepatic detoxification systems including glutathione S-transferase activity, cytochrome b5 levels and reduced thiol (SH) levels were enhanced in all the treatment groups, except for those maintained at the 600 mg/kg bw/day diet for 10 days. Significant reductions in cytochrome P450 and radiation-induced malondialdehyde levels were detected in all groups after 30 days of exposure. The authors concluded that the effects observed on the phase II glutathione S-transferase activity could at least in part be ascribed to eugenol. The level of 0.5% clove powder in the diet amounts to about 750 mg clove powder /kg bw and 600 mg eugenol/kg bw/day. This intake can be compared to subchronic studies carried out with eugenol itself. A study from Trubek laboratories (1958) in which 10 male and 10 female rats were given 89.7 mg eugenol/kg bw for 12 weeks without any adverse effects was reported [169] .

In another study [163] groups of 10 male and female rats were fed diets containing 0, 0.1 and 1.0% eugenol (amounting to doses of 0, 50 and 500 mg eugenol/kg bw) for 19 weeks without any adverse effects on growth rate, haematology, organ weights and histology of major tissues, pointing at a No-Observed-Adverse-Effect Level (NOAEL) of at least 500 mg eugenol/kg bw/dayIn a series of NTP (National Toxicology Program) studies [172] in which male and female F-344 rats were fed various levels of eugenol in the diet, amounting to 0.6, 1.25, 2.5, 5 and 10% for 14 days (resulting in doses of 300, 625, 1250, 2500 and 5000 mg eugenol/kg bw/day) and 0, 0.08. 0.15, 0.3, 0.6 and 1.25% for 90 days (resulting in doses of 0, 40, 75, 150, 300 and 625 mg eugenol/kg bw/day) there appeared to be a dose-related reduction in weight gain and mortality in the high dose groups of the 14 day study. In the 90 day study there were no compound-related effects on gross or microscopic pathology but a 12% reduction in weight gain in the high (625 mg eugenol/kg bw/day) dose group relative to the controls.

6.2.5. Chronic effects of clove extracts and eugenol on animals

There are no chronic toxicity studies on clove derived material, but there are long-term studies for its major constituent eugenol. No carcinogenicity was found in several studies in CD-1 mice dosed with 0 or 0.5 % eugenol in the diet (equivalent to 0 or 750 mg eugenol/kg bw/day) for 12 months, or with 0 or 2.5 μmol of eugenol (0 or 20-410 mg eugenol/kg bw depending on the bw) twice a week by gavage from 4 days of age till 35 days of age subsequently maintaining the animals without dosing for 14 months [107, 169]. Other adverse effects were not reported. Eugenol was also found to be not carcinogenic [172] in male F-344 rats given 0.3 % or 0.6 % eugenol in the diet (equal to 150 or 300 mg eugenol/kg bw/day) for 103 weeks or in female rats receiving diets containing 0.6 % or 1.25 % eugenol (resulting in doses of 300 or 625 mg/kg bw/day) for 103 weeks

Based on these and other studies the JECFA concluded that most of the available evidence indicates that eugenol is not carcinogenic and that the level causing no toxicological effect was 250 mg/kg bw in the diet for the rat study. This No-Observed-Adverse-Effect Level (NOAEL) of 250 mg/kg was used to derive an acceptable daily intake (ADI) applying an uncertainty factor of 100 (10 for interspecies extrapolation and 10 for intraspecies extrapolation) resulting in an ADI for eugenol at 0-2.5 mg/kg bw/day [169]

6.2.6. Evaluation of the safety in use of clove extracts as botanical pesticide in cultivation of pepper berries

As the human data are derived from case studies on clove oil for which no accurate exposure levels were presented, these data cannot be used as a starting point for risk assessment of the clove based botanical pesticides. Also animal data on the toxicity of clove-derived material is limited. The major adverse effect upon exposure to high levels of clove-based material seems to be hepatic failure, with some case studies in man also reporting depression of the nervous system, pulmonary oedema and acidosis and studies in experimental animals reporting damage to the stomach epithelium and pulmonary oedema in addition to hepatotoxicity. Since most studies point at a significant role for the major constituent eugenol in the adverse effects of clove-based materials, risk assessment of the clove-based botanical pesticides can be based on the risk assessment available for this food flavouring for which an ADI of 0-2.5 mg eugenol/kg bw/day has been established [169]. Assuming an eugenol level of the clove based botanical pesticide of 880 g/kg (88%), the highest level reported for clove based oil preparations [158] and a level higher than the value between 61 and 627 g/kg reported by the Phytochemical and Ethnobotanical Databases, it can be calculated that this ADI of 2.5 mg eugenol/kg bw/day would be equivalent to an estimated safe dose (ESD) of 2.8 mg clove oil/kg bw/day.

This value gives an indication of the range in which a safe dose for daily human exposure to clove-based extract or oil could be found. For a 2 year old 15 kg child this would imply a safe daily intake of 42 mg clove oil/day, which is 120 to 240 times lower than the acute oral doses of 5-10 ml (5-10 gram) clove oil reported in case studies to result in severe adverse health effects.

Clove or its extracts can be applied as mulch around the plant to kill root knot nematodes, sprayed as pesticide to fight above ground pests and mixed with the pepper berries to protect them during storage. The application as a mulch is not expected to result in a significant exposure of the consumer since the eugenol is not extracted and sprayed but the clove is just dried and put on the ground around the plant root.

In the application of clove extract to protect stored food a usual concentration of 2000 mg extract/kg food [11] is used amounting to 1600 mg eugenol/kg food. In a worst case situation where the extracts and its ingredients do not biodegrade, a daily consumption of 0.33 g of black pepper by a person of 60 kg [173] would result in an intake of 9 μg eugenol/kg bw/day.

For spraying clove extract it can be assumed that about 50mg extract/ml will be diluted 100 times to 0.5 mg extract/ml of which the farmers normally will use 500 ml/plant equivalent to 250 mg extract/plant. A worst-case estimate is that about 10% of the sprayed solution reaches 2 kg berries per plant (the average normal berry production per plant in Indonesia). The maximum residue of clove extract on pepper berries immediately after spraying therefore is expected to be 12.5 mg/kg berries. If the extracts and its ingredients would not biodegrade at all a daily consumption of 0.33 g of black pepper by a person of 60 kg [173], would result in an intake of 0.07 μg extract/kg bw/day which is equivalent to 0.056 μg eugenol/kg bw/day for an extract containing 80% eugenol [11],

Compared to an ADI for eugenol of 2.5 mg/kg bw/day a worst case intake of 9 μg eugenol/kg bw/day via stored berries or of 0.056 μg eugenol/kg bw/day via sprayed berries would result in a margin of safety of 2.8×10^2 and 4.5×10^4 respectively. Thus it can be concluded that the use of clove oil and its eugenol content as a botanical pesticide in pepper plantations is not of safety concern for the consumer.

6.3. Tuba root

Tuba root (Figure 9a) is mainly cultivated in the tropics for its roots as a source of the insecticide rotenone (Figure 9b). Rotenone occurs in a large number of leguminous plants, but for commercial use, it has primarily been derived from the roots of Tuba root species (not only *D. elliptica* but also *D. longicarpa* and *D. mallasensis*) from Southeast Asia and Lonchocarpus species (*L*onchocarpus *urucu, L. nicou* and *L.utilis*) from South America. The commercial material derived from Lonchocarpus is termed cube, barbasco nekoe or timbo while that of *Derris elliptica* is called derris root or tuba root [174)]. Rotenone is a commonly used pesticide and it is neurotoxic, especially for dopaminergic neurons. In research on Parkinson disease rotenone is used to induce Parkinson-like symptoms in laboratory animals [93, 175, 176)].

Rotenone inhibits the mitochondrial respiratory chain [177)], and by inhibiting mitochondrial complex I (NADH ubiquinone reductase) it causes apoptosis through enhancing mitochondrial reactive oxygen species production, ATP depletion and cellular anoxia [178, 179)]. Reports on effects of Tuba root-derived extracts in man or animals are limited and therefore the safety assessment was based also on reported studies for other rotenone-containing plant materials and rotenone.

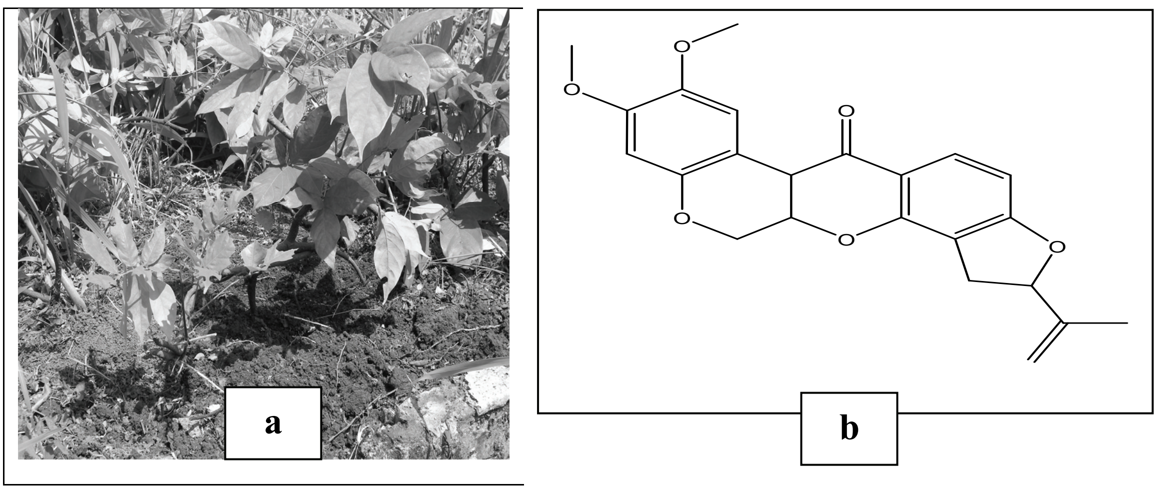

Figure 9. Tuba root (*D. elliptica*) plant (a) and structure formula of rotenone (b).

6.3.1. Effects of tuba root extracts on humans

Table 22 summarizes the reported effects of tuba root-derived material on humans upon oral exposure. These studies are limited to case reports of accidental or deliberate oral exposure. Upon oral intake, gastrointestinal absorption of the active compound rotenone is reported to be slow and incomplete [180)]. The irritant effect of rotenone on mucous membranes induces vomiting [180)] and this may further reduce the changes on systemic exposure upon oral intake.

It has been reported that deliberate ingestion of plant roots containing rotenone was common among individuals in Papua New Guinea who wanted to commit suicide [181)]. De Wilde *et al*. (1986) reported a case of accidental rotenone poisoning in a 3.5 year old girl (15 kg) with fatal outcome due to cardiopulmonary arrest upon ingestion of 10 ml of a botanical pesticide formulation corresponding with 40 mg rotenone/kg bw. It is to be noted that besides the 6.1% rotenone content this formulation also contained 18.5% oil of cinnamon, 27.5% oil of clove (this means an eugenol intake from 10-100 mg/kg bw, amounting to 4 to 40 times the ADI for eugenol), 17.5% oil of fir, 1.0% oil of rosemary and 1.0% oil of thyme). It is particularly noteworthy that the insecticide was labelled as “Natural product-Non toxic” The post-mortem rotenone concentration in stomach and blood were reported to be respectively 1260 and 2.4 mg/kg [177)]. De Wilde *et al*. reported the lethal dose to be 300-500 mg kg/bw.

A case study was described [182)] with a 47 years old diabetic women for whom deliberate ingestion of 200 ml of a 0.8% commercial rotenone solution derived from tuba root resulted in vomiting, unconsciousness and coma combined with severe liver dysfunction and metabolic acidosis, finally leading to death. The estimated intake of rotenone that can be calculated from these data assuming a body weight of 60 kg amounts to 25 mg/kg bw.

A fatal case of rotenone toxicity from *Pachyrhizus erosus* (Yam bean) occurred in Thailand where a 59 year old man was found dead after ingestion of seeds from yam bean[182)]. The authors ascribed the toxic effects to rotenone and detected the rotenone concentration in the gastric content and blood of the victim. The victim ate over 100 Yam bean seeds and died within 2 hours from congestive heart failure and respiratory failure. Adverse effects observed post-mortem were characteristic microscopic haemorrhage in the brain, lungs, liver and adrenal glands. The dose amounted to about 100 seeds estimated to be 100 gram of seeds [183)]. Assuming that the seeds contain 0.5% rotenone [182)] and an estimated dose of 500 mg this equals 8 mg rotenone /kg bw for a 60 kg person.

Overall these studies indicate that the acute oral toxicity of Tuba root is probably due to its rotenone content, and they also reveal that oral toxicity in man resulting in vomiting, unconsciousness, coma and death can already be observed at doses around 8-25 mg rotenone /kg bw. Human fatalities are reported to be rare, perhaps because rotenone is usually sold in low concentrations (1-5% formulation) [184)] because its absorption from the intestines is relatively slow and because its irritating action causes prompt vomiting.

Table 22. Effects of tuba root (*D. elliptica)* derived and other rotenone containing plant materials on humans

Plant part	patient	Administration	Dosages	duration	Observed effects	reference
0.8% rotenone solution (Bio liquid Derris Plus)	47 year diabetic woman 64 kg	Ingestion of 200 ml	25 mg/kg bw	One dose	Vomiting, unconscious, coma, death. Liver disfunction and severe metabolic acidosis (pH 7.09)	[177)]
Gallicide (botanical pesticide mix of 6.1% rotenone, 18.5% cinnamon oil, 27.5% clove oil, 17.5 % fir oil, 1% rosemary oil, 1% thyme oil	3.5 year old girl 15 kg	Swallowed a mouthful (ca 10 ml) of gallicide	40 mg/kg	One dose	Cardiopulmonary arrest leading to death within one day. Post mortum rotenone conc. 1260 mg/kg in the stomach, 2-4 mg/kg in blood, liver and kidney	[181)]
Yam bean seeds (0.5 % rotenone) [183)]	59 years old man	Ingestion of about 100 seeds (100 gram)	8 mg/kg bw	One dose	Microscopic hemorrhages in brain, lungs, liver and adrenal glands, death due to respiratory failure	[185)]

6.3.2. Effects of tuba root extracts on animals

The effects of Tuba root and other rotenone containing plant materials on animals are summarized in Table 23.

6.3.3. Acute effects of tuba root extracts and rotenone on animals

The oral lethal dose of rotenone reported for non-human mammals amounts to 13 mg to 1500 mg/kg bw [184, 186, 187]; varying from 13 to 130 mg/kg bw in guinea pigs [186-189] from 25 to 132 mg/kg bw in rats [184, 190] and 1500 mg/kg bw in rabbits [180] indicating that, compared to these species, humans are relatively sensitive for rotenone. Due to the low bioavailability upon oral intake [175] lethal doses upon intraperitoneal or intravenous administration are generally much lower but they are not relevant for estimation of the safety of Tuba root-derived rotenone containing material to be used as a pesticide resulting in oral exposure.

6.3.4. Subchronic effects of tuba root extracts and rotenone on animals

Subchronic studies on rotenone toxicity were performed in rats after subcutaneous administration of 3 mg/kg bw/day for respectively 14 or 28 days [93, 175, 176, 179, 191] In these studies rotenone appeared to cause neurotoxicity. This observation matches with the fact that in research on Parkinson disease rotenone is used to induce Parkinson-like symptoms in laboratory animals [192]. In a 2 year rat study in which rats were fed diets containing rotenone at doses up to 2.5 mg/kg bw/day no pathological changes were observed that could be attributed to rotenone [192]. Dogs fed rotenone for six months at doses up to 10 mg/kg/day had reduced food consumption and therefore reduced weight gain. At the highest dose, blood chemistry was adversely affected, possibly due to gasto-intestinal lesions and chronic bleeding [193]. In both studies a NOAEL of 0.4 mg rotenone/kg bw/day has been established [192, 194, 195].

Reproductive effects were detected in pregnant rats fed 10 mg/kg bw/day on days 6 through 15 of gestation. Effects observed were decreased number of live fetuses/dam, increased fetal resorption, and low birth weight accompanied by high maternal mortality already at 2.5 mg/kg bw/day [195]. These results are in accordance with those of others [40]. Again a NOAEL of 0.4 mg rotenone/kg bw/day was reported. In a re-registration evaluation [190, 192-194, 196, 197] the EPA reported a NOAEL of 0.375 mg/kg/day for chronic dietary exposure.

6.3.5. Chronic effects of tuba root extracts and rotenone on animals

There are no chronic toxicity studies on Tuba root derived material. The data on carcinogenicity, genotoxicity and teratogenic action of rotenone are inconclusive [40].

In a 6-month rotenone feeding study with dogs a NOEL was established of 0.4 mg/kg/day; the LOEL was 2 mg rotenone/kg/day with as main effects decreased mean body weight, decreased hematocrit and hemoglobin levels, decreased cholesterol, total lipids, and glucose

Table 23 Effects of tuba root (*D. elliptica*) derived and other rotenone containing materials on animals

Plant part	Animal	Administration	Dosages	Duration	Observed effects	Reference
Rotenone dissolved in glycol	guinea pigs	oral	50-100 mg/kg		NOAEL=50 mg/kg LOEL=75 mg/kg (Minimal LD)	[184)]
Rotenone dissolved in glycol	rabbits	oral	0.4-2.0 gram/kg		LOAEL=1.60 gram/kg (Minimal lethal dose)	[184)]
Rotenone dissolved in glycol	dogs	oral	0.1-1 g/kg		LOAEL > 1 gram/kg (Minimal lethal dose)	[186)]
Rotenone dissolved in olive oil	rats	oral	10 -1000 mg/kg	one dosage	NOAEL for mortality =10 mg/kg LD_{50}= 25mg/kg LD_{100}>35 mg/dkg	[187)]
	guinea pigs		7.5-25 mg/kg		LD_{50}=12 mg/kg LD_{100}=25 mg/kg NOAEL =7.5 mg/kg	
Rotenone dissolved in ethylene glycol	Guinea pigs	oral	50-200 mg/kg		Ratio dead/exposed animals 0/8 at 75 mg/kg	[189)]
	Rats	oral	50-200 mg/kg		8/10 at 200 mg 2/10 at 50 mg/kg 10/10 at200 mg/kg	
Rotenone dissolved in acetone	Wistar rats	oral	range unknown dose was 0,5-2 ml /kgbw)	one dosage	LD_{50}=60 mg/kg	[195)]
Rotenone suspended in corn oil	Pregnant Female Wistar rats (175-225 g)	orally (esopha-geal intubation)	2.5, 5.0 and 10.0 mg/kg /day	day 6 -15 of pregnancy on day 22 of pregnancy the dams were killed	LD_{60} =10mg/day (dams) LOAEL (fetuses) = 5mg/day (skeletal abnormalities) NOAEL =2.5 mg/kg/day	[184, 190, 198, 199)]

levels in the blood. Also an increased incidence of emesis and diarrhea was observed. (U.S.Fish and Wildlife Service, 1980). In a 2-year feeding study for oncogenic effects in rats effects observed at 3.8 mg/kg/day included reduced body weight in males and females, reduced food consumption in females, lower total protein and albumin levels in the blood, increased blood urea nitrogen levels, and increased incidences of adrenal gland angiectasis and hemorrhage (U.S. Fish and Wildlife Service, 1985). These results are in line with the results described for the semi-chronic experiments indicating the irritating nature of rotenone. Based on these studies recently a chronic reference dose (cRfD) for rotenone was defined by the US Environmental Protection Agency (EPA) at 0.0004 mg/kg bw/day[193)]. The cRfD is an estimate of a daily oral exposure for a chronic duration (up to a lifetime) to the human population that is likely to be without an appreciable risk of deleterious effects during a lifetime.

6.3.6. Evaluation of the safety in use of tuba root extracts as botanical pesticide in cultivation of pepper berries

The JECFA has not yet reviewed rotenone and established an ADI. Nevertheless; given the use of rotenone as a pesticide, some countries have limits for food products which amount to 0.04-0.1 mg/kg food [183)].

Rotenone containing tuba root extract can be sprayed as pesticide to fight above ground pests. As the seeds contain about 0.5% rotenone [173)] we assume that an extract of Tuba root contains 5% of rotenone, which would be equivalent to 50 mg rotenone/ml. If this formula is diluted 100 times to 0.5 mg rotenone/ml of which the farmers normally will use 500 ml/plant this is equivalent to 250 mg rotenone/plant. A worst-case estimate is that about 10% of the sprayed solution reaches 2 kg berries per plant (the average normal berry production per plant in Indonesia). The maximum residue level of rotenone on pepper berries immediately after spraying would then be 12.5 mg/ kg berries. If this would not degrade a daily consumption of 0.33 g of black pepper [11)] would result in an intake for a 60 kg person of 0.07 µg rotenone /kg bw/day.

Compared to the cRfD for rotenone of 0.0004 mg/kg bw/day this would imply a margin of safety of 5.7. When applying Tuba root extract to protect stored berries, about 2000 mg extract/kg food would be used [173)]. Assuming that the concentration of rotenone in the extract was about 0.5% amounting of 14 mg rotenone/kg food in which the extracts and its ingredients would not biodegrade at all, a daily consumption of 0.33 g of black pepper by a person of 60 kg [200)] would result in an intake of 0.08 µg rotenone/kg bw/day. This would result in an exposure 5 fold below the cRfD from 0,0004 mg/kg bw/day.

From this it can be concluded that the use of tuba root as botanical pesticide in cultivation of pepper berries would not be of safety concern for the consumer of these berries.

6.4. Sweet flag

Sweet flag (Figure 10a) grows in North America, Europe and Asia and its dried roots (Figure 10b) are known under the name calamus. Depending on the district of growth the root contains different amounts of a volatile oil [200]. One of the ingredients in this volatile oil is beta-asarone [201] (Figure 10c). The Council of Europe Committee of Experts in Flavouring Substances (CEFS) evaluated beta-asarone as an active principle in calamus-based food flavourings in 1981 and 1998[201, 202]. The CEFS concluded that beta-asarone is clearly carcinogenic in rodents and potentially genotoxic and that it would be prudent to reduce the levels of beta-asarone as far as possible. CEFS encouraged the use of *A. calamus* varieties with low contents or free of beta-asarone and proposed limits of 0.05 mg beta-asarone/kg for foods and beverages and 0.5 mg beta-asarone/kg for alcoholic beverages traditionally flavoured with calamus [203]. In addition CESF also indicated that the plant is considered as unfit for human consumption in any amounts. JECFA recommended that the oil of calamus used in foods should have the lowest practicable levels of beta-asarone and did not establish an ADI for beta-asarone [204].

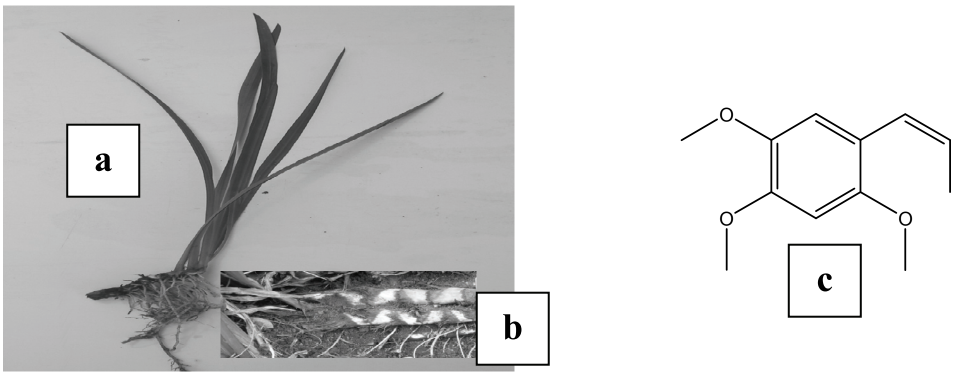

Figure 10. Sweet flag (a), roots (b), and structure formula of beta-asarone (c)

In 2002 the Scientific Committee on Food also evaluated the presence of beta-asarone in flavourings and other food ingredients with flavouring properties [204]. They concluded that, given the fact that beta-asarone shows a weak carcinogenic effect in rats and that the genotoxic potential of beta-asarone cannot be ruled out, the existence of a threshold cannot be assumed and a safe exposure limit could not be established. Consequently the SCF concluded that limitations in exposure and use levels were indicated [205, 206].

6.4.1. Effects of sweet flag extracts on humans

The roots and rhizomes of Sweet flag have been widely used in the Ayurvedic system of medicine for the treatment of neurosis, insomnia, melancholia and hysteria as well as for the treatment of diarrhoea, neurasthenia and epilepsy [204]. However, case studies on adverse effects in man have not been reported. Also no data have been identified on the toxicity of beta-asarone, the bioactive ingredient of. Sweet flag extracts, in humans [207)].

Vargas *et al*. 1998 [160)] reported a case of a 19 year old man who ingested 20 cm of sweet flag root with water. Four hours after ingestion the patient was presented to the emergency unit of a hospital. He was pale, diaphoric and vomiting a yellow liquid. Laboratorium testing showed a mild leucocytosis. After short intravenous treatment with saline and promethazine he was discharged in good condition 4 hours later. There were no adverse effects or sequelae observed in a one year follow-up check.

6.4.2. Effects of sweet flag extracts and beta-asarone on animals

Tables 24 present an overview of toxicity data of Sweet flag extracts or beta-asarone on animals

6.4.3. Acute effects of sweet flag extracts on animals

Several authors report LD_{50} values for beta-asarone or calamus oil containing beta-asarone from different sources in rats, mouse and/or guinea pigs (Table 24). An oral LD_{50} of calamus oil in young adult Osborne-Mendel rats of 777 mg/kg bw [208)]. And an oral LD_{50} of calamus oil in rats of 8880 mg/kg bw [203)]. JECFA also mentions data from an unpublished study from Taylor in [204)] reporting an oral LD_{50} of 4331 and 3497 mg/kg bw respectively for Kashmir and European calamus oil. Given that these oils contain approximately 5% beta-asarone [203)] these LD_{50} values amount to 216 mg beta-asarone /kg bw and 175 mg beta-asarone/kg bw respectively.

JECFA reports for beta-asarone an oral LD_{50} of 1010 mg/kg bw in rats [204)]._LD_{50} values upon intraperitoneal exposure are not included in the table as these are not relevant for exposure via the oral route. Altogether it can be concluded that calamus oil and beta-asarone show LD_{50} values upon oral exposure that range from 777 (Jammu variety) to 8880 mg calamus oil/kg bw and 200 to 1000 mg beta-assarone/kg bw and are of moderate to low acute oral toxicity.

6.4.4. Subchronic effects of sweet flag extracts and beta-asarone on animals

Table 5 presents an overview of subchronic toxicity studies performed with calamus oil or beta-asarone. These studies were discussed in detail in the SCF opinion on beta-asarone, and together point at a range of adverse effects on heart and liver, including for example proliferation of bile duct epithelium, portal area fibrosis with haemosiderin deposition, myocardial degeneration characterised by varying degrees of necrosis of muscle fibers, early fibrosis and infiltration with mononuclear cells [163)]. In a study where young adult male and female rats were exposed to 1.0 % calamus oil in the diet for 18 weeks (equivalent to about 1000 mg oil/kg bw/day) growth depression, increased mortality

Table 24 Effects of sweet flag *(A. calamus)* derived and other β-asarone containing materials on animals

Plant part	Animal	Adminis-tration	Dosage	Duration	Observed effects	Reference
β-asarone	rat	oral		acute	LD50=1010 mg/kg bw	[203]
Calamus oil	rat	oral		acute	LD50= 8880	[203]
Calamus oil Jammu variety*	rat	oral		acute	LD50= 777 Tremors, weight loss, "scawny appearance"	[203]
Calamus oil Kashmir variety*	rat	oral		acute	LD50= 4331	[203]
Calamus oil European variety*	rat	oral		acute	LD50= 3497	[163, 203]
Calamus oil Jammu variety*	10 male and 10 female rats	oral	0-1.0% in the diet (equivalent to 0-1000mg/kg bw	18 weeks	NOAEL=0.25 % Depressed growth of rats (at >0.25%). Dose dependent pathology in liver and heart.	[203]
Hydro alcoholic extract of calamus rhizome European variety*	Groups of 11 male and 11 female rats	Oral	0-5.3% in the diet (β-asarone] in diet 0-0,06%) (equivalent to 0-58,3 mg β-asarone/kg bw	13 weeks	No observed effects in the groups dosed with the European variety	Unpublished data from Weinberg 1969 reported in [203]
Jammu oil of Calamus (JOC)			0.1% in the diet (β-asarone in diet 0.07%) (~ 71 mg β-asarone/kg bw		Growth depression	
β-asarone and Jammu oil of calamus as control	Groups of 25 male and 25 female rats	oral	0, 0.02, 0.08 or 0.2% in the diet (equivalent to 0-100 mg β-asarone /kg bw/day) Positive control group: 0.25 % JOC in the diet (125 mg JOC/kg bw/day)	For two years	LD100=0.2% (0,2% in the diet ~ 100 mg B asarone/kg bw/day). NOAEL=0.02% ~ 10 mg B asarone/kg bw/day. Pathology: abdominal fluid, pleural cavities, liver and kidney changes. Tumors in small intestine. Atrophy of cardiac muscle cells, cardiac fibrosis. Fatty degeneration; infiltration, thrombosis	Taylor pers. comm. in [203]

Plant part	Animal	Adminis-tration	Dosage	Duration	Observed effects	Reference
Jammu oil of calamus	Groups of 25 male and 25 female rats	oral	0, 0.05, 0.1, 0.25 and 0.5% in the diet (0-250 mg JOC /kg bw/day	For two years	Dose dependent mortality (females dying earlier than males) severely reduced growth rates in the males at doses > 0.25% Liver and heart damage Fluid in pleural and/ or peritoneal cavity and tumours in intestines	Taylor pers. comm. in [203]
Jammu oil of calamus	Groups of 25 male and 25 female rats	oral	0, 0.005, 0.01 and 0.5% in the diet (equivalent to 0-250 mg/kg bw /day)	For two years	NOAEL=0.01% LC_{100}=0.5%. One leiomyosarcoma is observed. Increase heart and liver only in 0.5% dose group.	Taylor pers. comm. in [203]
European oil of calamus	Groups of 25 male and 25 female rats	oral	0.1, 0.5, 1.0 and 2.0% in the diet (equivalent to 0-1000 mg/kg bw/day)	2 years	NOAEL=<0.1% Leiomyosarcoma and hepatocellular adenomas in the 1.0 and 2.0% group. Dose dependent hepatic changes	Taylor pers. comm. in [206]
Extract of calamus rhizome	Male Swiss Albino mice with castor oil induced diarrhea.	oral	0., 3, 7.5 and 15 mg either in 0.1 ml water or methanol		Antidiarrhoeal activity methanolic- > aqueous extract.	[209]
β-asarone	rats and cats				Abolishment of the conditioned ovoidance in rats and reduced social behaviour of cats	[203] cited in SCF 2002

*. ß-asarone content of calamus oils varies with source of the plant. European and Kashmir variety containing approximately 5% ß-asarone. Indian Acorus calamus oil from the Jammu area contains approximately 75% ß-asarone[203].

and large amounts of clear fluid in the abdominal cavity were observed along with both macroscopic and microscopic effects on the liver and heart [203)]. Heart and liver damage were also reported upon 9 to 14 weeks oral exposure to dose levels of 1.0 % Jammu, European or Kashmir calamus oil in the diet or to 250 mg Jammu calamus oil/ kg bw/day, 847 mg European calamus oil/ kg bw/day or 1082 mg Kashmir calamus oil/ kg bw/day given by oral gavages. The severity of the heart and liver damage decreased in the order Jammu > European > Kashmir oil in the diet [210)].

6.4.5. Chronic effects of sweet flag extracts and beta-asarone on animals

In a 2 year study rats (25 males and 25 females for each group) were exposed to dietary levels of calamus oil (Jammu variety) of 0, 500, 1000, 2500 and 5000 mg/kg diet, amounting to approximately 0, 25, 50, 125 and 250 mg calamus oil kg bw/day [203)]. Moderate to marked growth depression was observed at all levels. Macroscopic and microscopic changes in liver tissue were observed as well as heart changes consisting of slight to moderate focal diffuse myocardial degeneration. After 59 weeks of exposure malignant tumours were found in the duodenal region of rats at all dose levels but were absent in the control group.

In another study male and female rats were orally exposed to 0, 0.1, 0.5, 1.0 and 2.0 % European calamus oil in the diet (equivalent to 0, 50, 250, 500 and 1000 mg oil /kg bw/day). Leiomyosarcoma and hepatocellular adenomas were reported in the two highest dose groups [210, 211)]. Also mice exposed to 52 mg beta-asarone/kg bw injected ip at day 1, 8, 15 and 22 and amounting to a total dose of approximately 1 mg, developed hepatomas one year later [210, 211)]. From all this it is concluded that high dosages of beta-asarone and beta-asarone containing oil from calamus are carcinogenic in rodents.

6.4.6. Evaluation of the safety in use of Sweet flag extracts as botanical pesticide in cultivation of pepper berries

Given the fact that calamus oil appeared to be carcinogenic in the long term studies with rats, and mice [204)]. and the fact that the genotoxic potential of beta-asarone cannot be ruled out [201-204)] it is not possible to derive a NOAEL from these (sub)chronic toxicity data. This implies that no safe level of exposure can be defined.

Various opinions on the safety evaluation of beta-asarone and/or the oil of calamus already concluded that beta-asarone is carcinogenic in rodents and potentially genotoxic and therefore it would be prudent to reduce the levels of beta-asarone in food as far as possible and that limitations in exposure and use levels are indicated[154)].

Applying the same assumptions to calculate the exposure of consumers of pepper berries as given above for Tuba root, the worst-case estimation is that the maximum residue level of Sweet flag extract on pepper berries immediately after spraying is 12.5 mg extract/ kg berries. Since the extract contains 65% beta-asarone [204] this is equivalent to 8.1 mg beta-asarone/kg berries. Without degrading a daily consumption of 0.33 g of black pepper/person would result in an exposure 0.07 μg extract/kg bw/day which is equivalent to 0.05 μg beta-asarone/kg bw/day. When applied to protect stored black pepper berries, 2000 mg calamus extract/kg food containing 65% of beta assarone amounts to 1300 mg beta-asarone /kg berries. Without degradation a daily consumption of 0.33 g of black pepper by a person of 60 kg would result in an intake of 0.43 μg beta-asarone/kg bw/day.

The maximum beta-asarone intake from all dietary sources has been estimated to amount to about 115 μg/ day [204] being about 2 μg/kg bw/day for a 60 kg person. Compared to this estimated daily intake, the intake of beta-asarone resulting from the use of Sweet flag extracts in the cultivation and storage of pepper berries would add significantly to the estimated daily intake from all other sources together. Beta-asarone has shown a weak carcinogenic effect in rats at dose levels of 20 mg/kg bw/day and higher[212]. From this it can be derived that the margin of exposure as compared to the worst case estimated intake resulting from the residual levels on sprayed pepper berries of 0.05 μg beta-asarone/kg bw/day will be higher than 4×10^5 and and for stored berries 4.7×10^4 thus higher than 10000. This indicates that the intake of beta-asarone from the pepper berries will be of low priority from a risk management point of view. However, the SCF concluded that the existence of a threshold cannot be assumed and a safe exposure limit cannot be established and limitations in exposure and use levels were indicated. Therefore, in spite of the fact that risks resulting from the use of Sweet flag as a botanical pesticide in cultivation of pepper berries would be limited if any, it has to be concluded that promoting the use of beta-asarone-containing calamus oil for use as a botanical pesticide should not be encouraged. Given the fact that the use of Sweet flag-based extracts as a pesticide does not relate to an unavoidable pollutant but to a deliberate use as botanical pesticide which can be avoided, it seems prudent to look for alternatives to be used as a botanical pesticide.

6.5. Pyrethrum

Extracts derived from the flowers of *C. cinerarieaefolium* (Figure 11a) also know as pyrethrum extracts, have been used as insecticides already for a long time. The active principles of these extracts are pyrethrin isomers, consisting of a mixture of predominantly three closely related esters of chrysanthemic acid (pyrethrins I) and three closely related esters of pyrethric acid (pyrethrins II) (Figure 11b). Pyrethroids are synthetic insecticides, chemically similar to pyrethrins found in natural pyrethrum extracts from the flowers of chrysanthemum.

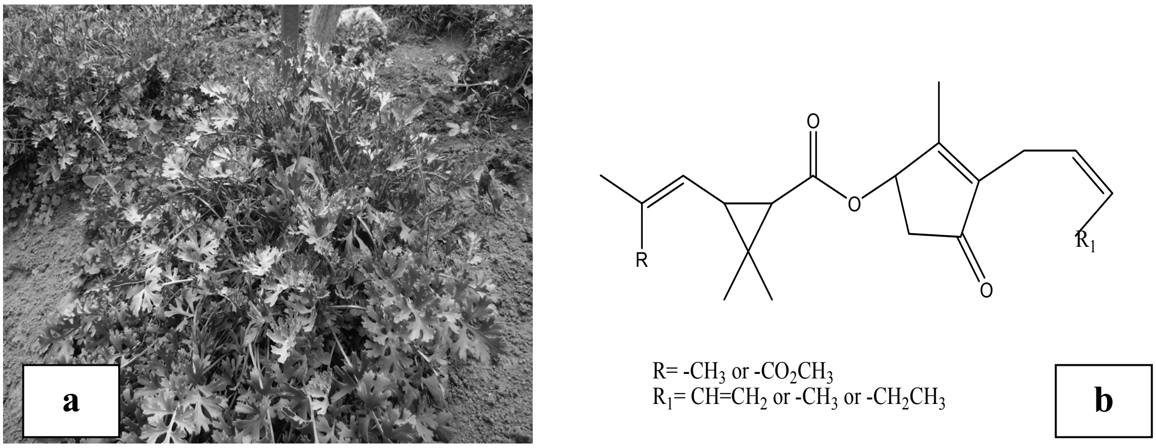

Figure 11. Pyrethrum (a), and structure formula of pyrethrins (b).

In insects pyrethrins and pyrethroids prolong the opening of the sodium channels, thereby producing stimulation of the nervous system that proceeds form excitation to convulsions and paralysis. Various reviews of the safety of pyrethrins and pyrethroids have been published including various toxicological assessments on pyrethrins by the joint meeting of the FAO Panel of Experts on Pesticide residues in food and the environment and the WHO Core assessment group (JMPR) [213] and the WHO evaluation on the safety of pyrethroids for public health use [212].

In 1999 the JMPR established an ADI for pyrethrum extract of 0-0.04 mg /kg bw and an acute reference dose (ARfD also called RfD) of 0-0.2 mg /kg bw [212]. The ADI was based on the NOAEL for liver damage in a 2 year rat study and a safety factor of 100 and the ArFD/RfD on the NOAEL for acute neurotoxicity in a rat study, also with a safety factor of 100.

Selection of varieties of *Chrysanthemum* rich in pyrethrins and optimisation of extraction techniques have resulted in pyrethrum extracts containing a total pyrethrin level up to 45-55% [213].

In addition ADI values for individual pyrethroids have been established that vary from 0.01 to 0.07 mg/kg/day ([180]. The present review, however, focuses on the pyrethrum extract containing the natural pyrethrins.

6.5.1 Effects of pyrethrum extracts and pyrethrins on humans

There are a few documented cases of fatal pyrethrin poisoning in man, although it has been concluded that the main toxicity to humans is related more to the solvent vehicle than to the pyrethrins themselves [214].

A 2 year old child died upon ingestion of 15 g of pyrethrum concentrate. An 11 month old baby whose mouth, nostrils and entire face were covered with pyrethrum powder had severe breathing difficulties, but these effects subsided within 1.5 hours [215)].

The American Association of Poison Control Centres (AAPCC) analysed incidents of human exposure to products containing pyrethrins and pyrethroids from 1994 to 1999. Unintended exposures were reported to involve nearly always (95% of reported cases) acute exposure and occurred mostly (93% of reported cases) around the home [215)]. Where there were symptoms, they were considered to be either unrelated to exposure or minor (38%), moderate (7.7%) or major effects (0.2%), with no deaths reported in the 49331 cases for which the medical outcome was known. It was also concluded that ingestion was often associated with minor effects because of very low levels of exposure, while inhalation and ocular routes were more likely to be related with a more severe medical outcome. Symptoms reported were especially ocular and dermal symptoms in children and a wider range of effects in adults including gastrointestinal, dermal, ocular, respiratory and neurological effects [215)], with especially respiratory and neurological symptoms occurring in cases with major adverse effects. For the JMPR in 2003 these data and outcomes of additional genotoxicity studies and studies on mechanism of action focussing on liver and thyroid tumorigenesis in rats, were no reason to modify the ADI and/or the ARfD/RfD of respectively 0-0.04 mg/kg bw/day and 0.2 mg/kg bw [212)].

6.5.2 Effects of pyrethrum extracts on animals

Table 25 presents an overview of the (sub) chronic toxicity data on *Pyrethrum* extracts on animals. The acute toxicity is discussed in the next section.

6.5.3 Acute effects of Pyrethrum extracts and pyrethrins on animals

In mammals pyrethrum has been reported to be only weakly toxic and oral LD_{50} values vary from 584 to 3900 mg pyrethrum extract/kg bw in rats, 273 to 796 mg pyrethrum extract/kg bw in mouse and > 2000 mg pyrethrum extracts/kg bw in rabbits [190)]. There is, however, a great divergence in the published literature with regards to acute toxicity of unpurified pyrethrum extract [212)].

6.5.4 Subchronic effects of Pyrethrum extracts and pyrethrins on animals

Table 26 presents an overview of the subchronic toxicity studies on pyrethrins described in detail by the JMPR [212)]. Oral studies in mice, rats, rabbits and dogs generally reveal the liver as the target organ. Reporting for example increased liver weight, increased incidence of hepatocellular hypertrophy, increased activities of plasma activities of aspartate and alanine aminotransferase, enlargement and congestion of the liver, with NOAELs of 1000 mg

Table 25. Effects of pyrethrum derived materials on animals

Plant part	Animal	Adminis-tration	Dosage	Duration	Observed effects	Reference
10% solution of pyrethrins in corn oil for male And 5% of pyrethrins solution in corn oil for female	Male and female Sprague - Dawley rats 15 animals /group	Oral gavages	0,40, 125 or 400 mg pyrethrins/kg bw for male 0, 20, 63 or 200 mg pyrethrins/kg bw for female	once	NOAEL = 20 mg/kg bw At high dosages 5 males and 2 females died on day of treatment, the other animals had tremors, urogenital area wetness, salivation, perinasal encrustation, exaggerated startle response, decreased grip strength, hind leg splay, increased body temp. Tremors also occurred in 3 of intermediate dose.	Hermansky, S.J. & Hurley, J.M. 1993 cited in [214, 216]
Pyrethrum	Rat Mouse	Oral	not specified	once	LD50= 584-900 mg/kg LD50=273-796 mg'kg	[212]
Extract containing 57.6% pyrethrins	Rat males or females	Oral	not specified	once	LD50 males= 2400-3900 mg/kg LD50 females=1000-1300 mg/kg NOAEL males= 710 mg/kg NOAEL females=320 mg/kg	[212]
Extract containing 57.6% pyrethrins	Charles River mice (groups of 15)	Oral	Males 47-1600 mg/kg/day Females 56-1800 mg/kg/day (original high dose was 4800 mg/kg/day for both sexes but animals died after 2-10 days)	13 weeks	4 males dosed 1600 mg/kg/day and 2 females dosed 1800 mg/kg/day died on day 2 . NOAEL= 160 mg/kg bw Increased incidence of hepatocellular hypertrophy at 460-1800 dose groups for both sexes.	Goldentahl 1988 Unpublished report cited by[212]

Plant part	Animal	Adminis-tration	Dosage	Duration	Observed effects	Reference
Extract containing 57.6% pyrethrins	Rats (groups of 15)	Oral in the diet	300-20000 mg/kg in the diet (equal to 17-1200 mg/kg/day for males and 22-1400 for females)	13 weeks	In the first week One maleof the1200 mg/kg bw /day, one female of the 700 mg/kg bw /day and 12 females of the 1400 mg/kg bw/day dose group died NOAEL= 57 mg/kg bw /day(1000 mg/kg diet)	Goldenthal 1988b Unpublished report cited by[212)]
Extract containing 57.6% pyrethrins	Dogs 2 male and 2 female/ dose	In the diet	600-6000 mg/kg diet (equal to 18-170 mg/kg bw/day for males and 19-200 mg/kg bw/day for females)	8 weeks	Clinical signs: in appetence, thin appearance, ataxia, trembling, oily coat, impaired limb function, shallow breathing, morbidity and death (1 male and both females of 6000 mg/kg diet group). NOAEL= 600 mg/kg diet (equal to 18 mg/kg/day) Effects in 1000 mg/kg diet (equal to 30 mg/kg bw/day) group were increased liver and increased testis weight.	Goldenthahl 1988c Unpublished report cited by[212)]
Extract containing 57.6 %pyrethrins	Groups of 28 male and female Charles River rats	In the diet	100-3000 mg/kg diet (equal to 10-300 mg/kg bw/day)	Two generation study F0 treated ≥ l 77 days before first of two mating. F1 treated ≥ 95 days before mating to produce the F2	NOAEL for parental and reproductive toxicity= 100 mg/kg diet In F1 reduction in food consumption at 3000 mg/kg diet and sporadically at 1000 mg/kg diet. Dose dependent reduced birth weight for the F1 males and all F2 pups and during lactation	Schardein 1989

Plant part	Animal	Administration	Dosage	Duration	Observed effects	Reference
Extract containing 57.6% pyrethrins suspended in 0.5% methylcellulose	25 mated female Charles River rats	Orally by gavage (3 ml /kg bw)	5-75 mg pyrethrins/kg bw/ day on day 6-16 of gestation	On day 20 of gestation the fetuses were removed surgically	NOAEL=75 mg/kg/day for maternal and development toxicity	(Schardein 1987 b)
Extract containing 57.6 %pyrethrins suspended in 0.5% methylcellulose	Groups of five inseminated female New Zealand white SPF rabbits	Orally by gavage (3 ml /kg bw)	37.5-600 mg peyrethrins /kg bw/day on day7-17 of gestation	On day 7-19 of gestation	At 600 mg/kg bw/day maternal deaths, tremors, convulsions, weight loss and fetal toxicity as high post implantation loss At 300mg/kg bw/day weight loss and tremors. No clear dose related effects in 37.5-150 mg/kg bw/day groups	Schardein 1987 c
Extract containing 57.6 % pyrethrins suspended in 0.5% methylcellulose	Groups of 16 inseminated female New Zealand white SPF rabbits	Orally by gavage (3 ml /kg bw)	25-250mg peyrethrins /kg bw/day	On day 7-19 of gestation. On day 29 of gestation the fetuses were removed surgically.	NOAEL= 25 mg/kg bw /day for maternal toxicity NOAEL= 250 mg.kg bw/day for developmental toxicity. Maternal body weight loss (high dose); slightly reduced body weight gain (intermediate dose). No treatment related fetal malformation	Schardein 1987 d
Extract containing 57.6% pyrethrins	Groups of 4 beagle dogs of each sex	In the diet	0-2500 mg/kg diet (equal to 2.6-66 and 2.8-75 mg/kg bw/day for males and females resp.	52 weeks	NOAEL=500 mg/kg diet (equal to 14 mg/kg/day) Increased liver weight for the males in the 2500 mg/kg group.	Goldenthal 1990a Unpublished reportcited by212)

Plant part	Animal	Adminis-tration	Dosage	Duration	Observed effects	Reference
Extract containing 57.6% pyrethrins	Groups of 60 male and female Charles River CD-1 mice	In the diet	100-5000 mg/kg diet (equal to 14-690 and 17- 830 mg/kg bw/day for males and females resp.	18 months	NOAEL=100 mg/kg diet Effects: dose related increased liver weights at 2500 and 5000 mm/kg diet plus microscopically vacuolar fatty changes in liver of the males. In treated males a not clearly dose dependent increase in incidence of alveolar bronchiolar carcinomas and treatment related increased incidence of longtumours	Goldentahl 1990b Unpublished report cited by212)
Extract containing 57.6% pyrethrins	Groups of 60 Charles River CD rats	In the diet	100-3000 mg/kg diet (equal to 4-120 mg/kg bw/day)	104 weeks	NOAEL =100 mg/kg diet in high dose group significantly reduced body weight during first 78 weeks coinciding with slightly decreased food intake, increased serum trans aminase activity in males. Increased incidences of benign tumours of liver, thyroid and skin, of follicular adenomas in females	Goldentahl 1990c Unpublished report cited by212, 214, 216)
Extract containing 57.6 %pyrethrins	Groups of 28 male and female Charles River rats	In the diet	100-3000 mg/kg diet (equal to 10-300 mg/kg bw/day)	Two generati-on study. F0 treated > l 77 days before first of two mating. F1 treated > 95 days before mating to produce the F2	NOAEL for parental and reproductive toxicity= 100 mg/kg diet In F1 reduction in food consumption at 3000 mg/kg diet and sporadically at 1000 mg/kg diet. Dose dependent reduced birth weight for the F1 males and all F2 pups and during lactation	Schardein 1989

Plant part	Animal	Adminis-tration	Dosage	Duration	Observed effects	Reference
Extract containing 57.6% pyrethrins suspended in 0.5% methylcellulose	25 mated female Charles River rats	Orally by gavage (3 ml /kg bw)	5-75 mg pyrethrins/kg bw/ day on day 6-16 of gestation	On day 20 of gestation the fetuses were removed surgically	NOAEL=75 mg/kg/day for maternal and development toxicity	(Schardein 1987 b)
Extract containing 57.6 %pyrethrins suspended in 0.5% methylcellulose	Groups of five inseminated female New Zealand white SPF rabbits	Orally by gavage (3 ml /kg bw)	37.5-600 mg peyrethrins /kg bw/day on day7-17 of gestation	On day 7-19 of gestation	At 600 mg/kg bw/day maternal deaths, tremors, convulsions, weight loss and fetal toxicity as high post implantation loss At 300mg/kg bw/day weight loss and tremors. No clear dose related effects in 37.5-150 mg/kg bw/day groups	Schardein 1987 c
Extract containing 57.6 % pyrethrins suspended in 0.5% methylcellulose	Groups of 16 inseminated female New Zealand white SPF rabbits	Orally by gavage (3 ml /kg bw)	25-250mg peyrethrins /kg bw/day	On day 7-19 of gestation. On day 29 of gestation the fetuses were removed surgically.	NOAEL= 25 mg/kg bw /day for maternal toxicity NOAEL= 250 mg.kg bw/day for developmental toxicity. Maternal body weight loss (high dose); slightly reduced body weight gain (intermediate dose). No treatment related fetal malformation	Schardein 1987 d

pyrethrum extract/kg diet amounting to 160 mg pyrethrum extract/kg bw/day in mice [212], and 57 mg pyrethrum extract/kg bw/day in rats [212]. The total pyrethrin content of these extracts was 57.6%. In other studies effects on the kidney were reported as well, including for example small focal or multifocal areas of tubular degeneration and regeneration in the renal cortex in rats at high dosages of 3000 and 10 000 mg pyrethrum extract/kg diet amounting to about 150 and 500 mg pyrethrum extract /kg bw/day [212]

6.5.5 Chronic effects of Pyrethrum extracts and pyrethrins on animals

The JMPR evaluation presents an overview of chronic toxicity studies and these are also summarised in Table 25. The ADI of 0-0.04 mg pyrethrum extract /kg bw is based on a NOAEL for liver damage in a 2 years rat study of 100 mg/kg in the diet amounting to 4 mg pyrethrum extract/kg bw and a safety factor of 100 [212]

6.5.6 Evaluation of the safety in use of pyrethrum extracts as botanical pesticide in cultivation of black pepper

Using the ADI for pyrethrum of 0.04 mg/kg bw/day established by the JMPR, and assuming 45-55% pyrethrins/mg extract (SCF 2002) this would be equivalent to 0.018-0.022 mg pyrethrins/kg bw. The ARfD (also called RfD) based on acute neurotoxicity of 0.2 mg/kg bw/day [169] is equivalent to about 0.09-0.11 mg. pyrethrins/kg bw. Safety evaluation of the use of extracts from chrysanthemum, known as pyrethrum extracts, as botanical pesticides can be based on these reference values for pyrethrum extract

Assuming Pyrethrum extract is sprayed as pesticide to fight above ground pests, and a formulation of Pyrethrum containing 50 mg of the extract/ml will be diluted 100 times to 0.5 mg extract/ml of which the farmers normally will use 500 ml/plant, this is equivalent to 250 mg extract/plant. As with the afore mentioned extracts a worst-case estimation is that 10% of the sprayed solution reaches 2 kg berries per plant resulting in a maximum residue of 12.5 mg Pyrethrum extract/kg pepper berries immediately after spraying. Without biodegradation a daily consumption of 0.33 g black pepper/60 kg person would result in an intake of 0.07 μg extract/kg bw/day. Since the extract contains 45-55% pyrethrins this would be equivalent to about 0.014 μg pyrethrins/kg bw/day.

Application of pyrethrum extract in the storage using 2000 mg extract/kg food containing 45-55% pyrethrin would amount to about 1000 mg pyrethrin/kg food and without biodegradation the estimated daily consumption of 0.33 g black pepper/60 kg person would result in an intake of 5.5 μg pyrethrin/kg bw/day or 11 μg pyrethrum extract/kg bw/day.

Compared to the ADI for pyrethrum of 0.04 mg pyrethrum extract/kg bw/day, equivalent to 0.018-0.022 mg pyrethrin/kg bw/day, an intake of 0.07 or 5.5 μg pyrethrum extract/kg bw/day, respectively through field application or application in the storage would imply a margin of safety of 570 and 7 respectively, this is 36 and 2857 fold below the ArfD/RfD of 0.2 pyrethrum extract /kg bw/day. From this it can be concluded that the use of Pyrethrum extracts as botanical pesticide in cultivation of pepper berries would not be of safety concern for the consumer of these berries.

6.6. Discussion and general conclusions

In this paper the safety-in-use for the consumer of four plant based botanical pesticides was evaluated. The plant-based botanical extracts evaluated included extracts from S. aromaticum (Clove), D. elliptica (Tuba root), A. calamus (Sweet flag) and *C. cinerarieaefolium* (Pyrethrum). In order to enable a comparison of the relative health risks of the different products, Table 7 gives an overview of the major outcomes of this safety assessment.

Of the four botanical pesticides evaluated only the Sweet flag extracts containing beta-asarone appears to be of possible concern because of this genotoxic and carcinogenic ingredient. Compared to the carcinogenicity data the margins of exposure calculated based on intake estimates based on proposed use and use levels of the botanical extracts were higher than 4 $x10^5$. From this it follows that the risk of application of Sweet flag extract would be low and would not be considered a high priority from a risk management point of view. However given the fact that for beta-asarone restrictions in use are indicated and the fact that use as a pesticide implies an avoidable risk, it is concluded that the use of this botanical pesticide should not be encouraged. Application as a mulch might be of no concern for the consumer since upon such applications the active ingredients are unlikely to reach the pepper berries and thus the consumer.

However, it should be stressed that the present review focussed only on risk for the consumer of the pepper berries cultivated or stored using the botanical pesticides. Evaluation of occupational risks upon inhalation, skin exposure or oral exposure upon inadequate handling of the material by farmers was not the objective of the present safety evaluation.

For Clove, most studies point at a significant role for the major constituent eugenol in the adverse effects of clove based materials, and therefore risk assessment of clove-based botanical pesticides was based on the risk assessment available for this food flavouring for which an ADI has been established at 2.5 mg eugenol/kg bw/day. Compared to the estimated intake of eugenol resulting from the use of clove-based extracts as botanical pesticide in the cultivation of pepper berries a margin of safety compared to the ADI for eugenol of $4x10^4$ can be derived for spraying in the field and $2.8x10^2$ for application in storage of berries. From this is can be concluded that the use of eugenol-containing clove-based extracts as botanical pesticide in the cultivation of pepper berries is not of safety concern for the consumer.

For Tuba root the safety assessment was based on its major active principle rotenone. The estimated safe daily exposure levels (cRfD rotenone = 0.0004 mg/kg bw/day)[11)] and the estimated intake of rotenone resulting from the use of Tuba root extracts as botanical pesticide in the cultivation of pepper berries results in a margin of safety of about 5. From this it can be concluded that the use of rotenone containing Tuba root based extracts is not a reason for concern for the consumer from the safety point of view.

The JMPR establised an ADI for pyrethrum, the active principle of Crysanthemum extracts containing pyrethrum isomers, of 0-0.04 mg/kg bw/day. Based on acute neurotoxicity data an ARfD/RfD of 0-0.2 mg/kg bw/day was indicated (JMPR 1999, 2003). Safety evaluation of the use of extracts from chrysanthemum, known as pyrethrum extracts, as botanical pesticides can be based on these reference values for pyrethrum extract.

Use of the pyrethrum extracts as botanical pesticide in pepper plantations and storage may result in estimated intake resulting from residual levels on berries that amount to 0.07 or 5.5 μg pyrethrum extract/kg bw/day, respectively leading to a margin of safety compared to the ADI of 570 and 7 for the sprayed and stored products respectively. From this is can be concluded that the use of Pyrethrum extracts as a botanical pesticide is not of safety concern for the consumer.

Altogether this leads to the conclusion that, whereas it seems prudent to look for other alternatives to be used as a botanical pesticide instead of *A. calamus* (Sweet flag) extracts containing beta-asarone, the use of *S. aromaticum* (Clove), *D. elliptica* (Tuba root) and *C. cinerarieaefolium* (Pyrethrum) derived pesticides in cultivation or storage of pepper berries should be encouraged. The *A. calamus* (Sweet flag) extract containing beta-asarone might still be considered for use as a botanical pesticide when applied as a mulch around the plant to kill root knot nematodes instead of spraying as a pesticide to fight above ground pests. The application as a mulch is not expected to result in a significant exposure of the consumer. Further safety assessment of such applications and also of the use of the other botanical pesticides evaluated in the present paper might include evaluation of the occupational risks for the farmers. At this moment it is not yet possible to estimate the occupational exposure (via skin and inhalation) of farmers spraying botanical pesticides, and occupational standards for botanical pesticides and their active principles generally have not been developed, so this part of the assessment of the safety-in-use of botanical pesticides remains an important topic for future research.

Table 26. Overview of final outcomes of the safety-evaluation for the consumers of black pepper treated with the plant-derived botanical pesticides tested before [217] either sprayed in the field or treated in storage.

Plant species as source for botanical pesticide	Active ingredient considered for risk assessment	Safe levels for plant-derived material or active ingredient	Estimated daily intake due to residues of the botanical pesticide used (worst case)	Safety evaluation
clove	eugenol	ADI 0-2.5 mg eugenol/kg bw/day equivalent to 2.8 mg clove oil/kg bw/day [40]	*Storage:* 9 µg eugenol/ kg bw/day *Sprayed*: 0.07 µg extract /kg bw amounting to 0.056 µg eugenol/kg bw/day	MOS of the stored product is $2.8x10^2$, while of the sprayed product is $4.5x10^4$ No safety concern
tuba root	rotenone	chronic reference dose (cRfD) 0.0004 mg rotenone /kg bw/day [204]	*Storage*: 0.08 µg rotenone/ kg bw/day *Sprayed*: 0.07 µg rotenone /kg bw 0.07µg extract/kg/day amounting to 0.0035 µg rotenone/kg/day	MOS in the storage and in the field of 5 and 5.7, respectively No safety concern
sweet flag	beta-asarone	Beta-asarone is genotoxic and carcinogenic (LOEC 20 mg/kg bw/day in rats)[212]	*Storage:* 0.43 µg beta asarone/ kg bw/day *Sprayed:* 0.07 µg extract /kg bw amounting to 0.05 µg beta-asarone/kg bw/day	Restrictions in use are indicated MoE > 4.7x104 .and $4x10^5$ .resp... Low priority for risk management. But use should not be encouraged
pyrethrum	pyrethrins	ADI 0-0.04 mg pyrethrum extract/kg bw (or 0.02 mg pyrethrin/kg bw/day), ARfD (also called RfD) of 0.2 mg pyrethrum extract /kg bw (0.10 mg pyrethrin/kg bw/day) [40]	*Storage:* 1.3 µg pyrethrin/ kg bw/day *Sprayed*: 0.07 µg extract /kg bw amounting to 0.056 µg eugenol/kg bw/day 0.07 µg extract/kg bw amounting to 0.00835 µg pyrethrins/kg bw/day	MOS is 7-570 resp. compared to ADI MOS is 140-11400 resp. compared to the ARfD No safety concern

Chapter

7
General Discussion

Abstract

The research described in this thesis investigated the effectiveness and safety of plant extracts as potential natural pesticides to be used as possible alternatives for synthetic pesticides that are intensively applied in black pepper plantations in Indonesia. The investigations started with a baseline field study into the current practice of pesticide use in black pepper plantations in Bangka Island. Subsequently laboratory, green house and field experiments were performed to assess the potencies of methanol extracts of 17 plants when used as botanical pesticides, also comparing their potential to that of a common synthetic pesticide to control pests of the pepper plant. A safety assessment for the consumer of black pepper treated with the most promising plant extracts was performed based on literature data from human and animal studies.

Overall, the results obtained confirm the hypothesis that botanical pesticides have the potency to be used to control pests of black pepper, providing a promising alternative for synthetic pesticide use, especially because they pose lower risks for the local environment.

7.1. Summary and discussion of the results

The baseline study (Chapter 2) showed that currently in pepper plantations in Bangka Island the farmers and their workers, and probably also the environment are at risk of high exposure to the synthetic pesticides applied. This is predominantly due to the unwise use of these pesticides that were used by 96% of the responding farmers of which 78% was not aware of the possible impact of the pesticides on their health. Most (69%) of the respondents were wearing daily clothes while spraying pesticides. About 18% of the respondents reported to have experienced pesticide poisoning. After spraying 100 pepper plants, the residues of pesticide in the urine and blood of 2 farmers that were monitored, increased by up to 10 and 1.5 fold respectively, indicating actual and direct exposure. The environmental risk assessment for the aquatic ecosystem and terrestrial below ground invertebrates using hypothetical scenarios indicated low risks for the terrestrial invertebrates but high risks for aquatic ecosystems. Fortunately, the residues of the major pesticides on pepper berries were below the maximum residue limits established for these synthetic pesticides. It is concluded that a main problem of the current usage of synthetic pesticides to control pests of black pepper results from the unwise ways of application. This includes too high concentrations being applied, continuously using a single brand of synthetic pesticide, poor application technology and hardly using protective equipment [33](Chapter 2). In order to minimize human and environmental health risks, alternative less toxic but comparably effective control strategies have to be promoted. The application of botanical pesticides is one approach that can help to solve these fundamental problems.

Botanical pesticides have been reported to have lower toxicity and a shorter degradation period compared to that of the synthetic pesticides [72] and therefore prevent the build-up of toxic residues in food chains and ecosystems [218]. Although some of the botanical extracts are highly toxic as well (e.g. tobacco) the right choice of extracts to be used could help to strongly reduce the health risks for farmers and the environment. A greater biodegradability of botanical pesticides also reduces the exposure of consumers, which is important for the competitive position of the local farmers on the critical International market for pepper berries. Western consumers increasingly prefer 'greener' products that are free of pesticide residues. Indonesian pepper berries have been rejected on the market before because the Food and Drug Administration (FDA) found too high residues of pesticides [11].

Another advantage for the local farmers of the use of locally produced botanical pesticides is that they can earn money by growing the plants used as raw material for production of the botanical pesticides. This will increase income and make them less dependent on expensive imported synthetic pesticides.

In subsequent laboratory experiments of the present thesis the potencies of methanol extracts of 17 plants when used as botanical pesticides were characterized. In a bioassay with the major underground pest of black pepper, *Meloidogyne incognita,* the extracts of clove (*Syzygium aromaticum)*, tobacco (*Nicotiana tabacum*), sweet flag (*Acorus calamus*), and betlevine (*Piper betle*) were shown to be most toxic against the nematode (Chapter 3). The shapes of the dead nematodes differed in a characteristic and consequent way between groups of plant extracts and pesticides, which may be an indicator for the mode of action of the tested pesticides.

The main pests of black pepper plants can be divided in 2 groups: a) under ground pests that attack roots of pepper plants and b) above ground pests that attack stems and canopy of the plant. In chapter 3 a green house bioassay was described in which mulching of the grounded clove bud was shown to be effective against the underground nematode (*Meloidogyne incognita*). It was also demonstrated that the nematicidal effect of the mulch was not significantly different from that obtained with the recommended synthetic pesticide carbofuran. It was concluded that the application of a mulch of the grinded clove bud is a promising control strategy for nematodes in the field (Chapter 3).

The potent nematicidal activity of the powder formulation of the clove bud is a promising finding for local farmers in Bangka Island, especially to control infestation of the root-knot nematode, *M. incognita*. This is the case because farmers currently indicate that there is no effective synthetic pesticide available to control this pest [219)](Chapter 2) and once a pepper plant is attacked by the nematode, it is impossible to kill this pest without also destroying the host [150)]. As eugenol, the active ingredient of the clove, also is a potent fungicide [23)], mulching also is expected to be effective to suppress the development of the root-rot disease of pepper caused by the mould *Phytophthora palmivora* [22)]. In addition mulching with organic material enriched with eugenol has been reported effective to control the stem-rot disease of vanilla caused by the fungus *Fusarium* sp. [3, 220)].

In additional laboratory bioassays with the model insect pest species *Tribolium castaneum*, the extracts of pyrethrum (*Chrysanthemum cinerarieaefolium*), sweet flag (*A. calamus*), tobacco (*N. tabacum*), lemongrass (*Cymbopogon citratus*) and clove (*S. aromaticum*) appeared to be most effective (Chapter 4). Further bioassays with these promising plant extracts showed that pyrethrum, sweet flag, and clove were the most potent extracts against *Lophobaris piperis.* In a next step the most effective extracts formed the basis for development of a new botanical formulation (Chapter 5). Chemical analysis revealed the presence of the active ingredients pyrethrin (1.8%), β-assarone (7.8%) and eugenol (4%) in the new formulation. The formulation was effective in laboratory assays against two major aboveground black pepper pests i.e. *Dasynus piperis* and *Diconocoris hewetti* and three minor aboveground black pepper pests i.e. *Aphis craccivora, A. gossypii*, and *Ferrisia virgata.* In subsequent field experiments the botanical formulation killed most pest species living on the pepper plants and appeared to be less toxic for the natural enemies of caterpillars, months, aphids, beetles also living on the treated plants than the recommended synthetic pesticide, deltamethrin. This means that the botanical formulation is more selective than the synthetic pesticide deltamethrin. Within nine hours after application of the botanical formulation the treated plants were successfully recolonized by ants and spiders pointing at efficient degradation of the active principles.

The effectivity of the formulation to control two major above ground pests of black pepper in the field i.e. *D. hewetti* and *D. piperis* [145)] is of great economical importance as these two pest species not only cause direct losses, but also indirect damage to the plants and berries. *D hewetti* feeds by sucking the inflorescence and the very young fruit bunches resulting in up to 39 % loss of pepper production [144)]. *D. piperis* sucks the fruit liquid content causing 5% direct reduction in pepper production [221)] but more serious is the secondary damage if the wounded berries then are infected by the fungus *Colletotrichum* causing berries-bunch-rot [146)] after which no berries can be harvested at all.

Due to the greater selectivity in combination with the lesser persistence, less side-effect are to be expected on untargeted species in the environment such as natural enemies and insect pollinators which are important to maintain the stability of agro ecosystems. Insect pollinators in plantations are crucial to ensure good berry production. Natural enemies help to keep populations of pests under their economic threshold thereby reducing the number of pesticide applications needed and thus reducing pest control cost further [125)].

The results suggest that botanical pesticides can be applied in pepper plantations to partially replace the use of 20 currently recommended synthetic insecticides. Of these synthetic pesticides several serious adverse effects to the farmers [133)], consumers [134)], live stocks [135)] and the environment [57)] have been reported. There is an estimation that annually about 3 million cases of pesticide poisoning occur worldwide with 220 000 deaths [3)].

Of course unwise use of botanical pesticides could be harmful. Therefore the safety of these pesticides has to be assessed as well. The effectiveness of the botanical pesticides chosen for the formulation developed in this study did not only depend on direct toxicity but also on repellency and anti-feeding action. And although they are not persistent some residues on the berries could not be excluded.

In the final chapter (Chapter 6) a consumer safety evaluation was performed of the application of the four botanical pesticides that might be used in pepper berry crop protection, including preparations from *Syzygium aromaticum* (Clove), *Derris elliptica* (Tuba root), *Acorus calamus* (Sweet flag), and *Chrysanthemum cinerarieaefolium* (Pyrethrum). Of the four plant extracts evaluated individually only the sweet flag extract was considered of possible concern because it contains beta-asarone which has been shown to have genotoxic and carcinogenic potencies. However, the margin of exposure calculated based on worst case intake estimates based on the proposed use of sweet flag extracts was higher than $4x10^5$ based on which the risk of application of sweet flag extract would be considered a low priority from a risk management point of view. However given the fact that for beta-asarone restrictions in use are indicated and the fact that the use as a pesticide implies an avoidable risk, it is concluded that the use of this botanical pesticide should not be encouraged. Application of sweet flag as a mulch will be of no concern for the consumer since upon such applications the active ingredients are unlikely to reach the pepper berries and thus the consumer. Also for the farmer such application would imply a much lower exposure than the use of an extract.

Overall, the results obtained confirm the hypothesis that botanical pesticides have the potency to be used to control pests of black pepper, providing a promising alternative for synthetic pesticides use, especially because they pose lower risks for the local environment.

7.2. Future perspectives

The increasing demand for healthy agricultural products for which absence of pesticides residues is a strong selling point will encourage the usage of botanical pesticides. In

Indonesia, development of botanical pesticides may be facilitated by the fact that the country provides a lot of natural plants that can be used as raw material.

Application of the clove bud powder as mulch to control the population of the root-knot nematode, *M. incognita*, one of the main pepper pests might also be of use for other commodities because the nematode is one of an important polyphagous pest species and has been reported damaging more than 700 plant species such as beans, cabbage, carrot, potato, pea, tomato, tea, cereals, sugarcane, tobacco and ornamental plants [3)].

The emulsified formulation developed in the present thesis containing extracts of pyrethrum, clove and sweet flag, is also expected to be effective against other important pest species related to the ones tested. In this study the emulsified formulation has been proven effective against *A. gossypii, A. craccivora, F. virgata*, and *Valanga nigricornis,* which are polyphagous pest species [3)]. For example, *A. gossypii* and *A. craccivora* have been reported attacking cacao, citrus, coffee, cotton, kapok, potato, rosella, sesamum, and tea. *F. virgata* has been known as a major pest of cacao, citrus, coffee, jute, and many vegetables and ornamental plants. *V. nigricornis* has been reported attacking bamboo, banana, cacao, cassava, coconut, coffee, cotton, jack fruit, kapok, maize, mango, paddy, richinus, rubber, sorghum, sugar cane, and sweet potato . In short, the emulsified formulation developed in the present thesis also have the potential to control pests of many horticultural, ornamental, and industrial crops. When residues for consumers are to be expected, the effectiveness of the formulation without sweet flag could be tested, to avoid exposure to beta-asarone.

7.3. Main conclusions

The health and environmental risks of synthetic pesticides in pepper plantations on Bangka Island predominantly arise from unwise use and low awareness of the associated risks. Therefore a change in habits and safer pesticides are needed.

1) Botanical pesticides pose lower risks for human and environmental health than chemical pesticides because the mode of action of botanical pesticides is often based on their repellent activity and inhibition of feeding and not on direct toxicity.
2) Mulch of clove bud is a potent botanical approach to fight the most problematic underground pest of black pepper, the root-knot nematode (*M. incognita*), and equally effective as the currently recommended synthetic pesticides.
3) The emulsified botanical pesticide formulation containing extracts of pyrethrum, sweet flag and clove is very effective to control two main above ground pepper pests *D. piperis*, and *D. hewetti*. For the third major black pepper pest, *L. piperis*, another approach has to be developed.
4) Although decreased health risks are to be expected for the farmer and the environment when synthetic pesticides are being replaced by botanical pesticides, this still has to be studied further.
5) Based on safety assessment no health risk is to be expected for the consumer of pepper berries treated with the botanical formulation.

References

1) S. Deciyanto, P. Wahid, D. Manohara and A. Dhalimi, *International News* **XXII**, 39-42 (1998).
2) C. Indrawanto and A. Wahyudi (1996) Supply and demand of white and black pepper. Bogor, Indonesian Spice and Medicinal Crops Research Institute, pp. 180-187. (Indonesian).
3) L. G. E. Kalshoven, "The Pest of Crops in Indonesia," Pt. Ichtiar Baru Van-Hoeve, Jakarta, 1981.
4) Z. A. Siddiqui, A. Iqbal and I. Mahmood, *Applied Soil Ecology*, **16**, 179-185 (2001).
5) Disbun Propinsi Bangka Belitung (2004) The development of pests, diseases and weeds. Annual Report of the province of Bangka-Belitung. Bangka. (Indonesian).
6) R. F. Davis and O. L. May, *Crop Sci*, **45**, 2312-2317 (2005).
7) D. Soetopo and E. A. Wikardi, *Industrial Crops Research Journal* **2**, 22-25 (1989).
8) Wiratno and D. Soetopo, *Bulletin Research Institute for Spice and Medicinal Crops*, **5**, 115-120 (1990).
9) Department Pertanian, "Pesticides for Agriculture and Estate Crops," Koperasi Ditjen Bina Sarana Pertanian, Jakarta. (Indonesian), 2005.
10) R. J. Tobin, *Food Policy*, **21**, 211-228 (1996).
11) Wiratno, D. Taniwiryono, P. V. d. Brink, I. M. C. M. Rietjens and A. J. Murk, *Journal of Environmental Toxicology*, **22**, 405-414 (2007).
12) R. Z. Yang and C. S. Tang, *Economic botany*, **42**, 376-406 (1988).
13) I.-K. Park, J.-Y. Park, K.-H. Kim, K.-S. Choi, I.-H. Choi, C.-S. Kim and S.-C. Shin, *Nematology*, **7**, 767-774 (2005).
14) N. K. Sangwan, B. S. Verma, K. K. Verma and K. S. Dhindsa, *Pesticide Science*, **28**, 331-335 (2004).
15) B. J. Vayias, C. G. Athanassiou and C. T. Buchelos, *Crop Protection*, **25**, 766-772 (2006).
16) G. H. Schmidt and M. Streloke, *Journal of Stored Products Research*, **30**, 227-235 (1994).
17) R. Biebel, E. Rametzhofer, H. Klapal, D. Polheim and H. Viernstein, *International Journal of Pharmaceutics*, **256**, 175-181 (2003).
18) M. M. Rahman and G. H. Schmidt, *Journal of Stored Products Research*, **35**, 285-295 (1999).

19) S.-I. Kim, J.-H. Yi, J.-h. Tak and Y.-J. Ahn, *Veterinary Parasitology*, **120**, 297-304 (2004).
20) W. Thorsell, A. Mikiver and H. Tunon, *Phytomedicine*, **13**, 132-134 (2006).
21) M. Tombe, D. Wahyono and I. G. R. Purnayasa (2003) Controlling white root disease of cashew Bogor, Indonesian Spice and Medicinal Crops Research Institute, pp. 1-15. (Indonesian).
22) M. Tombe, A. Nurawan and Sukamto, Utilization of clove leaves to control the stem-rot disease of vanilla, Results of the experiments on the usage of botanical pesticides, Bogor, Indonesia, Indonesian Spice and medicinal Crops Research Institute, 1993, p. 28-35. (Indonesian).
23) P. M. Mahon and A. Purwantara (2004) Major Crops affected by Phytophthora, ACIAR Monograph 114. Available: http://www.aciar.gov.au/web.nsf/att/JFRN-6BN9E3/$file/mn114-part2.pdf. Last cited: July 16th, 2007.
24) A. Kardinan, *Journal of Spice and Medicinal Crops*, **3**, 37-40 (1995).
25) B. C. R. Zhu, G. Henderson, F. Chen, F. H and R. A. Laine, *Journal of Chemical Ecology* **27**, 1617-1625(1619). (2001).
26) C. G. Athanassiou and N. G. Kavallieratos, *Crop Protection*, **24**, 703-710 (2005).
27) S. H. Ho, L. P. L. Cheng, K. Y. Sim and H. T. W. Tan, *Postharvest Biology and Technology*, **4**, 179-183 (1994).
28) S. S. Nathan, K. Kalaivani, K. Sehoon and K. Murugan, *Chemosphere*, **62**, 1381-1387 (2006).
29) B. T. Niber, *Journal of Stored Products Research*, **30**, 297-301 (1994).
30) T. Mardiningsih and A. Kardinan, *Journal of Spice and Medicinal Crops*, **3**, 41-44 (1995).
31) A. H. Wardhana, S. P. W. Kumarasinghe, L. Arawwawala and L. S. Arambewela, *Indian Journal of Dermatology* **52**, 43-47. (2007).
32) H. N. Opolot, A. Agona, S. Kyamanywa, G. N. Mbata and E. Adipala, *Crop Protection*, **25**, 1145-1152 (2006).
33) C. Regnault_Roger, *Integrated Pest Management Reviews* **2**, 25-34 (1997).
34) B. J. R. Philogène, C. Regnault-Roger and C. Vincent (2005) Botanicals: yesterday's and Today's promises, ed. C. Regnault-Roger, B. J. R. Philogène and C. Vincent. USA, Lavoisiern publishing Inc, pp. 1-15.
35) I. Albar (1997) Indonesian National Parks Homepage: Indonesian biodiversity. , Available: http://www.geocities.com/RainForest/4466/biodiver.htm. Last visited: June 7th, 2007.

36) A. Kardinan, "Botanical Pesticide; Formulation and application," PT. Penebar Swadaya, Jakarta, Indonesia. (Indonesian), 2002.
37) K. Tuley de Silva, "A Manual on the Essential Oil Industry," United Nations Industrial Development Organization, Vienna, 1996.
38) K. P. P. Nair, "The agronomy and economy of black pepper (*Piper nigrum* L.) the "king of spices". ," Vol. 82., Academic Press, New York, 2004.
39) S. Deciyanto, P. Wahid, D. Manohara and A. Dhalimi (1998) Adoption of a good agricultural practice to prevent contamination on an Indonesia experience, vol. 22, pp. 39-42.
40) Department of Agriculture of Indonesia, "Pestisida untuk pertanian dan perkebunan," Koperasi Ditjen Bina Sarana Pertanian. , Jakarta. (Indonesian). 2005.
41) J. Jeyaratnam, *World Health Statistics Quarterly*, **43**, 139-144 (1990).
42) P. J. Van den Brink, M. M. S. Ter Horst, W. H. J. Beltman, J. Vlaming and H. Van den Bosch (2005) PRIMET version 1.0, manual and technical description. A Decision Support System for assessing Pesticide Risks in the tropics to Man, Environment and Trade. . Wageningen, The Netherlands., pp. 1185.
43) European Union, *Official Journal of the European Union*, **C.240**, 1-23. (1997).
44) H. Van den Bosch, L. Chaowen, Pham Van Hoi, M. M. S. Ter Horst, P. J. Van den Brink, P. Yunliang, J. G. Groenwold, C. Yibing, N. Van Dung, M. S. Van Wijk and J. Vlaming (2006) Environmental risks of pesticide use in intensive vegetable farming systems in peri-urban Hanoi (Dong Anh) and Chengdu (Pengzhou) Wageningen, The Netherlands., pp. 1285.
45) D. De Zwart (2002) Observed regularities in species sensitivity distributions for aquatic species, ed. L. Posthuma, G. Suter and T. Traas. Boca Raton, FL, USA, Lewis Publishers, pp. 133-154.
46) G. K. Frampton, S. Jänsch, J. J. Scott-Fordsmand, J. Römbke and P. J. Van den Brink, *Environmental Toxicology and Chemistry*, **25**, 2480-2489. (2006).
47) Dinas Perkebunan Propinsi Bangka Belitung (2004) The development of pests, diseases and weeds. Annual Report of the province of Bangka-Belitung. (Indonesian).
48) Department Pertanian, "Pesticides for Agriculture and Estate Crops," Koperasi Ditjen Bina Sarana Pertanian. p 490. (Indonesian), Jakarta., 2005.
49) Department-Pertanian, "Pestisida untuk pertanian dan perkebunan," Koperasi Ditjen Bina Sarana Pertanian. , Jakarta. (Indonesian). 2005.
50) Disbun-Propinsi-Bangka-Belitung (2004) The development of pests, diseases and weeds. Annual Report of the province of Bangka-Belitung. Indonesian.

51) Pesticide Residue Committee (2006) Maximum residue levels (MRL), Available; http://www.pesticides.gov.uk/prc.asp?id=956#Where_can_I_obtain_more_information_on_MRLs (Cited: September 01st, 2006).
52) Staatscourant, *231*, 15-30. p. (2002).
53) Pesticide-Residue-Committee (2006) Maximum residue levels (MRL). Available; http://www.pesticides.gov.uk/prc.asp?id=956#Where_can_I_obtain_more_information_on_MRLs (Cited: September 01st, 2006).
54) G. Rainbird and D. O'Neill, *Applied Ergonomics*, **26**, 187-193 (1995).
55) C. Wilson and C. Tisdell, *Ecological Economics*, **39**, 449-462 (2001).
56) D. C. Little, A. Yakupitiyage, K. Satapornvanit, K. Kaewpaitoon, T. Ungsethaphand, G. K. Milwain, D. J. Baird, P. J. Van den Brink, G. T. Taylor and A. J. A. Nogueira (2003) Managing Agrochemicals in Multi-use Aquatic Systems (MAMAS). State-of-the-system report: . Thailand, Asian Institute of Technology and Kasetsart University, Bangkok.
57) F. Konradsen, W. van der Hoek, D. C. Cole, G. Hutchinson, H. Daisley, S. Singh and M. Eddleston, *Toxicology*, **192**, 249-261 (2003).
58) H. Murphy, A. Sanusi, R. Dilts, M. Djajadisastra, N. Hirschhorn and S. Yuliatiningsih, *Journal of Agromedicine*, **6**, 61-85 (1999).
59) M. Kishi, N. Hirschhorn, M. Djajadisastra, L. N. Satterlee, S. Strowman and R. Dilts, *Scan J Work Environ Health* **21**, 124-133 (1995).
60) D. Koh and J. Jeyaratnam, *The Science of the total environment*, **188 Suppl 1**, S78-S85 (1996).
61) J. Harris, "Chemical Markets, Health Risks and Residues," CABI Publishing, New York, 2000.
62) World Health Organization (1978) Data sheets on pesticides No. 33: chlorpyrifos-methyl.
63) K. Satapornvanit, D. J. Baird, D. C. Little, G. K. Milwain, P. J. Van den Brink, W. H. J. Beltman, A. J. A. Nogueira, M. A. Daam, I. Domingues, S. S. Kodithuwakku, M. W. P. Perera, A. Yakupitiyage, S. N. Sureshkumar and G. J. Taylor, *Australasian Journal of Ecotoxicology* **10**, 85-95 (2004).
64) S. Maxwell (1999) The meaning and measurement of Poverty, 3 Edition. London, UK Available; http://www.odi.org.uk/publications/briefing/pov3.html (Cited January 28th 2007).
65) P. M. Chapman, *International Journal of Environment and Pollution* **13**, 351-356 (2000).
66) A. D. Sagar, *Environmental impact assessment review*, **11**, 257-279 (1991).

67) K. R. Kranthi, D. R. Jadhav, S. Kranthi, R. R. Wanjari, S. S. Ali and D. A. Russell, *Crop Protection*, **21**, 449 (2002).
68) D. J. Ecobichon, *Toxicology*, **160**, 27 (2001).
69) L. P. Pedigo, S. H. Hutchins and L. B. Higley, *Annual Review Entomology* **31**, 341-368 (1986).
70) S. Tjitrosemito (1993) Integrated management of paddy and aquatic weed in Indonesia. Bogor, BIOTROP. p 19. Available; http://www.agnet.org/library/eb/367b/ (Cited; November 29th, 2006).
71) P. C. Matesson, *Annual Review of Entomology* **45**, 549-574 (2000).
72) C. Regnault-Roger (2005) New insecticides of plant origin for the third millenium?, ed. B. J. R. Regnault_Roger, C. Philogene and C. Vincent., Lavoisier Publishing Inc. , pp. 17-35.
73) Z. Khan and Y. H. Kim, *Applied Soil Ecology*, **35**, 370-379 (2007).
74) Z. Khan, Y. H. Kim, S. G. Kim and H. W. Kim, *Bioresource Technology*, **98**, 69-73 (2007).
75) H. Okada and H. Harada, *Applied Soil Ecology*, **35**, 582-598 (2007).
76) V. M. Williamson and A. Kumar, *Trends in Genetics*, **22**, 396-403 (2006).
77) M. Browning, D. B. Wallace, C. Dawson, S. R. Alm and J. A. Amador, *Soil Biology and Biochemistry*, **38**, 401-404 (2006).
78) C. Fernandez, R. Rodriguez-Kabana, P. Warrior and J. W. Kloepper, *Biological Control*, **22**, 103-114 (2001).
79) N. Javed, S. R. Gowen, M. Inam-ul-Haq, K. Abdullah and F. Shahina, *Crop Protection*, **In Press, Corrected Proof** (2006).
80) I. Ujvary, "Pest control agents from natural products, Handbook of Pesticide Toxicology," Academic Press. San Diego, San Diego, ed. 2, 2001.
81) S. Orme and S. Kegley (2006) PAN Pesticide Database, Pesticide Action Network, vol. 2007. North America.
82) European Food Safety Authority (2006) Conclusion regarding the peer review of the pesticide risk assessment of the active substance: carbosulfan ed. -. Summary of the EFSA Scientific Report (2006) 91, finalised: 28 July 2006. Available: http://www.efsa.europa.eu/etc/medialib/efsa/science/praper/conclusions/carbosulfan.Par.0008.File.dat/praper_concl_sr91_carbosulfan_summary_en.pdf. Last visited: May, 15th 2007.
83) S. Yuliani and S. Rusli, "Extraction of botanical pesticides," Indonesian Spice and Medicinal Crops Research Institute. Bogor, 2003.

84) K. R. Barker (1985) Nematode extraction and bioassays, ed. K. R. Barker, C. C. Carter and J. N. Sasser, vol. 2. Raleigh, NC, N.C. State Graphics, pp. 19-35.
85) D. Collett, "Modeling binary data," Chapman and Hall, London, 1991.
86) M. Akhtar, *Bioresource Technology*, **46**, 255-257 (1993).
87) M. M. Mackeen., A. M. Ali., M. A. Abdullah., R. M. Nasir., N. B. Mat., A. R. Razak. and K. Kawazu., *Pest Management Science*, **51**, 165-170 (1999).
88) D. D. W. a. R. G. Davide (1998) The root-knot nematode of banana. Musa Pest Factsheet No. 3. Available: http://bananas.bioversityinternational.org/files/files/pdf/publications/pest3_en.pdf. Last visited: April 26th 2007.
89) M. P. Perez, J. A. Navas-Cortes, M. J. Pascual-Villalobos and P. Castillo, *Plant Pathology*, **52**, 395-401 (2003).
90) J. Varma. and N. K. Dubey. (1998) Prospectives of botanical and microbial products as pesticides of tomorrow. Available: http://www.ias.ac.in/currsci/jan25/articles22.htm. Last visited April 26th 2007.
91) Y.-H. W. Feng., *Huazhong Agricultural University Journal* **3**, 235-238 (2001).
92) M. Akhtar, *Applied Soil Ecology*, **7**, 219-223 (1998).
93) S. A. Tiyagi and M. M. Alam, *Bioresource Technology*, **51**, 233-239 (1995).
94) M. Abid, University of Karachi (1996).
95) A. A. Adegbite. and S. O. Adesiyan., *World Journal of Agricultural Sciences*, **1**, 18-21 (2005).
96) A. K. Onifade and B. Fawole, *Global Journal of Pure and Applied Sciences*, **2**, 9-15 (1996).
97) International Centre for Research in Agroforestry Trees in agricultural syatems. Available: http://www.echotech.org/technical/az/aztext/azch4tre.htm#Table. Last visited May 10th, 2007.
98) R. Pandey, A. Kalra, S. Tandon, N. Mehrotra, H. N. Singh and S. Kumar, *Journal of Phytopathology*, **148**, 501-502 (2000).
99) M. Akhtar and I. Mahmood, *Bioresource Technology*, **48**, 189-201 (1994).
100) V. T. Nguyen., L. L. Hall., G. Gallacher., A. Ndoye., D. L. Jolkovsky., R. J. Webber., R. Buchli. and S. A. Grando., *Journal of Dental Research*, **79**, 939-949 (2000).
101) J. T. Debell., *The Quarterly Review of Biology*, **40**, 233-251 (1965).
102) M. Isman (2005) Problems and opportunities for the commercialization of botanical insecticides, ed. C. Regnault-Roger, B. Philogène and C. Vincent.
103) E. Zuskin, A. Bouhuys and M. Saric, *Clinical Allergy*, **11**, 243-248 (1981).

104) Z. Begum, S. S. Shaukat and I. A. Siddiqui, *Plant Pathology Journal* **2**, 174-180 (2003).

105) K. H. Wang, R. McSorley, A. J. Marshall and R. N. Gallaher, *Applied Soil Ecology*, **27**, 31-45 (2004).

106) M. Akhtar and A. Malik, *Bioresource Technology*, **74**, 35-47 (2000).

107) M. S. Nahar, P. S. Grewal, S. A. Miller, D. Stinner, B. R. Stinner, M. D. Kleinhenz, A. Wszelaki and D. Doohan, *Applied Soil Ecology*, **34**, 140-151 (2006).

108) I. H. Mian and R. Rodriguez-Kabana, *Nematropica*, **12**, 205-220 (1982).

109) R. Rodriguez-Kabana, G. Morgan-Jones and I. Chet, *Plant Soil*, **10**, 237-247 (1987).

110) T. A. Forge, E. Hogue, G. Neilsen and D. Neilsen, *Applied Soil Ecology*, **22**, 39-54 (2003).

111) G. W. Yeates, D. A. Wardle and R. N. Watson, *Soil Biology and Biochemistry*, **31**, 1721-1733 (1999).

112) Anonymous (2005) Price of clove decreased, farmers were suppressed by the living cost. .

113) R. W. Addor (1995) Insecticides, ed. C. Godfrey. New York, Marcel Dekker Inc pp. 1-63.

114) G. H. Schmidt, E. M. Risha and A. K. M. El-Nahal, *Journal of Stored Products Research*, **27**, 121-127 (1991).

115) S.-I. Kim, C. Park, M.-H. Ohh, H.-C. Cho and Y.-J. Ahn, *Journal of Stored Products Research*, **39**, 11-19 (2003).

116) S.-I. Kim, J.-Y. Roh, D.-H. Kim, H.-S. Lee and Y.-J. Ahn, *Journal of Stored Products Research*, **39**, 293-303 (2003).

117) K. J. Pradhan, P. S. Variyar and J. R. Bandekar, *Lebensm.-Wiss.U-Technol*, **32:121-123** (1999).

118) A. O. Oyedele, A. A. Gbolade, M. B. Sosan, F. B. Adewoyin, O. L. Soyelu and O. O. Orafidiya, *Phytomedicine*, **9**, 259-262 (2002).

119) S. Radhika, K. K. S and W. I. S, *Journal of Essential Oil Research*, **May/June 2006**, Available: http://findarticles.com/p/articles/mi_qa4091/is_200605/ai_n17174947 (Cited July2nd 2007) (2006).

120) P. P. Hien, H. Gortnizka and R. Kraemer, *Omonrice*, **11**, 83-92 (2003).

121) A. Oparaeke and O. Bunmi, *Archives of Phytopathology and Plant Protection*, **39**, 247-251(5) (2006).

122) J. de S. Luna, A. F. dos Santos, M. R. F. de Lima, M. C. de Omena, F. A. C. de Mendonca, L. W. Bieber and A. E. G. Sant'Ana, *Journal of Ethnopharmacology*, **97**, 199-206 (2005).

123) R. Jbilou, A. Ennabili and F. Sayah, *African Journal of Biotechnology*, **5**, 936-940 (2006).
124) S. Rusli, B. S. Sembiring, S. Suhirman, E. A. Wikardi, Tritianingsih and Wahyudiono (2003) Effect of storage on stability and effectivity of pyrethrum insecticides. Jakarta, Indonesian Spice and Medicinal Crops Research Institute, pp. 12 (Indonesian).
125) S. Dasgupta, C. Meisner, D. Wheeler, K. Xuyen and N. Thi Lam, *International Journal of Hygiene and Environmental Health* **210**, 121-132 (2007).
126) M. J. Pascual-Villalobos and A. Robledo, *Industrial Crops and Products*, **8**, 183-194 (1998).
127) M. Jensenius, A.-M. Pretorius, F. Clarke and B. Myrvang, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **99**, 708-711 (2005).
128) N. Raja, S. Albert, S. Ignacimuthu and S. Dorn, *Journal of Stored Products Research*, **37**, 127-132 (2001).
129) U. K. C. Devaraj, Ed. "Management of the American cockroach, *Periplaneta americana* L. (Blattidae: Dictyoptera) with plant seed oils, edited by Anthropology Conservation Laboratory, Smithsonian Institution. Ethnographic Conservation Newsletter. 18. Available: http://www.nmnh.si.edu/anthro/ConservL/ICOMnews/N18/icom1098.htm#NATURAL%20PRODUCTS. Last visited: May15th 2007," 1995.
130) M. B. Isman, *Pesticide Science*, **38**, 57-63 (1993).
131) K. Bauer, Garbe, D., Surburg, H (1990) Common fragrance and flavour materials 2nd Edition, VCH Verlagsgesellschaft Ltd., Weinheim pp. 153-155.
132) P. Koona, V. Tatchago and D. Malaa, *Journal of Stored Products Research*, **43**, 248-251 (2007).
133) K. Huffling, *Journal of Midwifery & Women's Health*, **51**, 19-25 (2006).
134) Y. Wang, P. Kruzik, A. Helsberg, I. Helsberg and W.-D. Rausch, *Forensic Science International*, **In Press, Corrected Proof** (2006).
135) S. Giacomazzi and N. Cochet, *Chemosphere*, **56**, 1021-1032 (2004).
136) G. Misra, S. G. Pavlostathis, E. M. Perdue and R. Araujo, *Appl. Microbial. Biotechnol*, **45**, 831-838 (1996).
137) Wiratno, D. Taniwiryono, I. M. C. M. Rietjens and A. J. Murk, (2008). Laboratory and field studies on the effectiveness of botanical extracts and a newly formulated botanical pesticide formulation against pests of black pepper . Submitted
138) J. Duval, (1993). Home production of pyrethrumIn: Hill S.B., (ed.).Available: http://www.eap.mcgill.ca/AgroBio/ab360-02e.htm. Last visited: March, 21st 2007

139) Association of Official Analytical Chemists, "Official methods of analysis of the Association of Official Analytical Chemists," Association of Official Analytical Chemists, Washington DC, ed. 13th 1980.
140) Y. Masada, "Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry," John Wiley & Sons Inc, New York, 1980.
141) M. Y. Hu, G. H. Zhong, Z. T. Sun, G. Sh, H. M. Liu and X. Q. Liu, *Journal of Applied Entomology*, **129**, 413-417 (2005).
142) B.-S. Park, S.-E. Lee, W.-S. Choi, C.-Y. Jeong, C. Song and K.-Y. Cho, *Crop Protection*, **21**, 249-251 (2002).
143) D. E. Simonet, W. I. Knausenberger, L. H. Townsend and E. C. Turner, *Archives of Environmental Contamination and Toxicology* **5**, 339-347 (1978).
144) Suprapto and Thomas, *Industrial Crops News*, **14**, 119-125 (Indonesian) (1989).
145) I. M. Trisawa, D. Soetopo, Sumarko and Sihwiyono, *Bulletin Research Institute for Spice and Medicinal Crops*, **7**, 6-10 (Indonesian) (1992).
146) W. Zhang and S. M. Swinton (2006) A natural enemies-adjusted economic threshold for pest control. Available: , http://www.ucl.ac.uk/bioecon/8th_paper/Zhang_Swinton.pdf. Cited: July 17th 2007.
147) PAN Pesticides Database. Northern house mosquito (*Culex pipiens*) Toxicity Studies - Toxicology studies from the primary scientific literature on aquatic organisms. Available: http://www.pesticideinfo.org/List_AquireAll.jsp?Species=210&Effect=. Last cited: March 3rd 2008.
148) PAN Pesticides Database. Chemical Toxicity Studies on Aquatic Organisms: Toxicity Studies for Deltamethrin on Insects - Toxicology studies from the primary scientific literature on aquatic organisms. Available: http://www.pesticideinfo.org/List_AquireAll.jsp?Rec_Id=PC33475&Taxa_Group=Insects. Last cited: May 11th 2008
149) Xiulan Li, Lei Ma, Lixin Sun and Changliang Zhu, *Applied Entomology and Zoology*, **37**, 305-308 (2002).
150) M. Serrano, D. Martinez-Romero, S. Castillo, F. Guillen and D. Valero, *Innovative Food Science & Emerging Technologies*, **6**, 115-123 (2005).
151) I. Mustika and A. R. Slamet, Efication of clove products and other botanical plants against nematodes attacking black pepper, Conference on Results of the experiments in order to utilize botanical pesticides, Bogor, Indonesia. (Indonesian), Indonesian Research Institute for Spice and Medicinal Crops, 1994.
152) I. Mustika, *Black pepper Monograph. Pp 130-141. (Indonesian)* (1996).
153) A. E. M. F. Soffers, Wiratno, A. J. Murk and I. M. C. M. Rietjens, (2008).

154) Wiratno, D. Taniwiryono, *B. S. Sembiring*, *I. M. Trisawa*, *I. M. C. M. Rietjens* and *A. J. Murk*, (2008). Laboratory and field studies on the effectiveness of botanical extracts and a newly formulated botanical pesticide formulation against pests of black pepper . Submitted
155) Tajuddin, S. Ahmad, A. Latif and I. A. Qasmi, *BMC Complement Altern Med*, **3**, 6 (2003).
156) J. S. Eisen, G. Koren, D. N. Juurlink and V. L. Ng, *J Toxicol Clin Toxicol*, **42**, 89-92 (2004).
157) USDA. Agricultural Research Service (2005) National genetic resources program. Phytochemical and Etnobotanical databases, National Germplasm Resources Laboratory, Beltsville, Maryland.
158) B. W. Lane, M. J. Ellenhorn, T. V. Hulbert and M. McCarron, *Hum Exp Toxicol*, **10**, 291-4 (1991).
159) G. Hartnoll, D. Moore and D. Douek, *Arch Dis Child*, **69**, 392-3 (1993).
160) P. M. Jenner, E. C. Hagan, J. M. Taylor, E. L. Cook and O. G. Fritzhugh, *Food and Cosmetic Toxicology*, **2**, 327-43 (1964).
161) H. A. Sober, F. Hollander and E. K. Sober, *Proc Soc Exp Biol Med*, **73**, 148-51 (1950).
162) J. M. Taylor, P. M. Jenner and W. I. Jones, *Toxicol Appl Pharmacol*, **84**, 378-87 (1964).
163) E. C. Hagan, W. H. Hansen, O. G. Fitzhugh, P. M. Jenner, W. I. Jones, J. M. Taylor, E. L. Long, A. A. Nelson and J. B. Brouwer, *Food Cosmet Toxicol*, **5**, 141-57 (1967).
164) E. C. Hagan, P. M. Jenner, W. I. Jones, O. G. Fitzhugh, E. L. Long, J. G. Brouwer and W. K. Webb, *Toxicol Appl Pharmacol*, **41**, 18-24 (1965).
165) JECFA (1967) Eugenol (FAO Nutrition Meetings Report Series 44a), Joint FAO/WHO Expert Committee on Food Additives.
166) M. V. Kumari, *Cancer Lett*, **60**, 67-73 (1991).
167) F. U. Lauber and F. Hollander, *Gastroenterology*, **15**, 481-6 (1950).
168) JECFA (1979) Eugenol (WHO food additive series 14), Joint FAO/WHO Expert Committee on Food Additives.
169) JECFA (1982) Eugenol (WHO food additive series 17), Joint FAO/WHO Expert Committee on Food Additives.
170) C. R. Hitchcock, *J Natl Cancer Inst*, **12**, 723-33 (1952).
171) K. J. Hartiala, M. Pulkkinen and P. Ball, *Nature*, **210**, 739-40 (1966).

172) NTP (1983) Carcinogenesis studies of eugenol inj F3444/N rats and B6C3F1 mice. , ed. U. S. D. o. H. a. H. services, National Toxicology Program.
173) K. U. Pradeep, P. Geervani and B. O. Eggum, *Plant Foods for Human Nutrition*, **44**, 137-148 (1992).
174) K. R. Solomon (2001) Ecotoxicological Risk assesment of Pesticides in tne environment, ed. R. Krieger, vol. 2, seond Edition. New York, Academic press.
175) P. Caboni, T. B. Sherer, N. Zhang, G. Taylor, H. M. Na, J. T. Greenamyre and J. E. Casida, *Chem Res Toxicol*, **17**, 1540-8 (2004).
176) V. N. Uversky, *Cell Tissue Res*, **318**, 225-41 (2004).
177) D. M. Wood, H. Alsahaf, P. Streete, P. I. Dargan and A. L. Jones, *Crit Care*, **9**, R280-4 (2005).
178) M. F. Molina-Jimenez, M. I. Sanchez-Reus, M. Cascales, D. Andres and J. Benedi, *Toxicol Appl Pharmacol*, **209**, 214-25 (2005).
179) T. B. Sherer, R. Betarbet, C. M. Testa, B. B. Seo, J. R. Richardson, J. H. Kim, G. W. Miller, T. Yagi, A. Matsuno-Yagi and J. T. Greenamyre, *J Neurosci*, **23**, 10756-64 (2003).
180) R. E. Gosselin, R. P. Smith and H. C. Hodge, "Clinical toxicology of Commercial Products.," Williams and Wilkins, Baltimore/London, ed. 5, 1984.
181) A. R. De Wilde, A. Heyndrickx and D. Carton, *J Forensic Sci*, **31**, 1492-8 (1986).
182) P. Narongchai, S. Narongchai and S. Thampituk, *J Med Assoc Thai*, **88**, 984-7 (2005).
183) F. Hosein, *Plant Molecular Biology Reporter*, **19**, 65a- 65e (2001).
184) H. B. Haag, *J Pharmacol Exp Ther*, **43**, 193-208 (1931).
185) E. A. Holland, *Transactions of the Royal Society of Tropical Medicine and Hygiene* **32**, 293-294 (1938).
186) H. D. Lightbody and J. A. Matthews, *Ind Eng Chem*, **28**, 809-811 (1936).
187) M. B. Shimkin and H. H. Anderson, *Proc Soc Exp Biol Med*, **34**, 135-138 (1936).
188) A. J. Lehman (1954) Chemicals in foods: a report of the association of food and drug officials on current developments Part ll . Pesticides. Section l. Introduction, vol. 15, pp. 122-123.
189) R. Santi and C. E. Toth, *Farmaco [Sci]*, **20**, 270-9 (1965).
190) D. Ray (1991) Pesticides derived from plants and other organisms, vol. 2, first Edition. New York, Academic press, pp. 585-636.
191) R. Betarbet, T. B. Sherer and T. Greenamyre, *BioEssays*, **24**, 308-318 (2002).
192) National research council (1983) Drinking Water and Health, vol. 5. Washington DC, National Academy Press, pp. 2-54.

193) IPCS (1992) Rotenone Health and Safety Guide No. 73. Geneva, World Health Organisation.
194) U.S. National Library of Medicine (1995) Hazardous Substances Databank. Bethesda, MD.
195) K. S. Khera, C. Whalen and G. Angers, *J Toxicol Environ Health*, **10**, 111-9 (1982).
196) U.S. Environmental Protection Agency, *Fed. Regist.* , **48**, 36246-36247 (1983).
197) National Toxicology Program (1984) Toxicology and Carcinogenesis Studies of Rotenone in F344/N Rats and B6C3F Mice. Bethesda, MD, National Institute of Health.
198) H. Kidd and D. R. James (1991) The Agrochemicals Handbook , Third Edition. Cambrige, UK, Royal Society of Chemistry information services.
199) M. J. Ellenhorn and D. G. Barceloux, "Medical Toxicology. Diagnoisis and treatment of human poisoning," Elsevier, New York, 1988.
200) W. Goggelmann and O. Schimmer, *Mutat Res*, **121**, 191-4 (1983).
201) CEFS (1998) Review of the toxicity of the active principles for the 4th edition part II of “flavouring substances and natural sources of flavouring”. Council of Europe Committee of Experts on Flavouring Substances, pp. 16-42.
202) CEFS (1993) Note to 32nd Session CoE, April 1993. UK, Council of Europe Committee of Experts on Flavouring Substances.
203) JECFA (1981) Monograph on B-asarone, Joint FAO/WHO Expert committee on food Additives.
204) SCF (2002) Opinion of the Scientific Committee on Food on the presence of Beta-asarone in flavourings and other food ingredients with flavouring
properties, ed. S. C. o. Food. Brussel, Eurppean commission, pp. 15.
205) P. C. Dandiya and H. Cullumbine, *J Pharmacol Exp Ther*, **125**, 353-9 (1959).
206) F. G. Shoba and M. Thomas, *Journal Of Ethnopharmacology*, **76**, 73-76 (2001).
207) C. P. Vargas, L. R. Wolf, S. R. Gamm and K. Koontz, *J Toxicol Clin Toxicol*, **36**, 259-60 (1998).
208) D. L. J. Opdyke, *Fd. Cosmet. Toxicol.*, **15**, 623-26 (1977).
209) J. D. Sharma, P. C. Dandiya, R. M. Baxter and S. I. Kandel, *Nature*, **192**, 1299-300 (1961).
210) J. M. Taylor, W. I. Jones, E. C. Hagan, M. A. Gross, D. A. Davis and E. L. Cook, *Toxicology and Applied Pharmacology*, **10** (1967).
211) R. W. Wiseman, E. C. Miller, J. A. Miller and A. Liem, *Cancer Res*, **47**, 2275-83 (1987).

212) JMPR (1999) Pesticides residues in food , Toxicological evaluations;Joint meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. Rome, WHO.

213) WHO (2005) Safetyof pyrethroids for public health use, Word health Organisation Pesticide evaluation Scheme (WHOPES).

214) W. J. Hayes, "Pesticides studied in man " Williams and Wilkins, Baltimore/London, 1982.

215) JMPR, (2003).

216) E. Y. Spencer (1982) Guide to the chemicals used in crop protection 7th edition. Ottowa Canada, Agriculture research institute.

217) Wiratno, D. Taniwiryono, I. M. C. M. Rietjens, B. S. Sembiring, I. MaraTrisawa and A. J. Murk, *Submitted* (2008).

218) D. Soetopo (1983) Pesticide Utilization on Black peppers in Indonesia. , vol. 17, pp. 25-26.

219) M. Guerena (2006) Nematodes: Alternative controls. National Sustainable Agriculture Information Service. Available: http://www.attra.org/attra-pub/nematode.html#intro. Last visited: June 15th, 2007, vol. 2007.

220) D. Soetopo, *Industrial Crops Research Journal*, **3**, 27-30 (1991).

221) D. Soetopo and Z. Asnawi, (1989). Pepper pest status in Bangka and its control. Technical Meeting of Pepper and Cacao in Bangka Island, August 7-8th 1989. Indonesian Spice and medicinal Crops Research Institute, Bogor. (Indonesian)

Summary

Black pepper (*Piper nigrum* L) is an important commodity of Indonesia, which has been cultivated since the 6th century. The plant plays an important role in local economies since 95% of the plantations are cultivated by smallholder farmers. Because of this important economic value, proper plant production is highly valued. One of the central factors to maintain plant production is how to control key pests of the plant such as the root-knot nematode, *Meloidogyne incognita,* the stem borer, *Lophobaris piperis,* the tinged bug, *Dasynus piperis*, and the bug, *Diconocoris hewetti* (Chapter 1). Currently, farmers habitually use synthetic pesticides to control these pests. However, this habit poses not only a serious health risk to local workers and the people living near the treated areas, but also threatens non-target species (Chapter 2). Therefore, it has become an important issue to find relatively easy alternative control strategies, which are comparable effective as the synthetic pesticides, but safer to the farmers, consumers, and the environment and available at low price. One of the possible alternatives would be the use of botanical pesticides. Indonesia seems to be in a good position to develop and utilize this pesticide since the country has a rich biodiversity of plant species. Nowadays, the increased consumer request in developed countries for organic products stimulates the interest in the use of botanical pesticides.

Chapter 3 describes the nematicidal activity of 17 plant extracts against the root-knot nematode, *M. incognita*. Results demonstrate that shapes of the dead nematodes in laboratory experiments can clearly be distinguished differed in a characteristic way, and groups of pesticides and plant extracts. This phenomenon may be an indicator for the modes of action of the tested pesticides. The green house experiment indicates that raw material of clove bud is comparable effective as the recommended synthetic pesticide. Chapter 4 describes contact toxicity, oral toxicity and repellency of 17 plant extracts against the model insect species *Tribolium castaneum*. This study shows that the most promising candidates for consideration as botanical pesticides are extracts of pyrethrum, sweet flag, tobacco, clove, lemongrass, neem, vetiver, graviola, citrosa and black pepper.

Formulation of three of 10 most potent extracts was developed and tested in the laboratory followed by field experiments. Laboratory experiments indicate that extracts from pyrethrum, sweet flag and clove show the highest toxicity and/or repellent effect toward *L. piperis*. Field experiments reveal that the formulation is able to control most pest species of the pepper plants in the meantime being less toxic towards the 11 monitored species of natural enemies for known pest organisms such as caterpillars, aphids, moths, beetles than that of the recommended synthetic pesticide, deltamethrin. Furthermore, the field experiments reveal that within 9 hours after application the treated plants are recolonized again by ants and spiders, indicating a short degradation period of the formulation.

Altogether it is concluded that the newly defined botanical formulation provides an effective and environmentally friendly alternative for controlling several pests of black pepper (Chapter 5).

The safety of five botanical pesticides i.e. pyrethrum, clove, sweet flag, and derris is evaluated for human oral exposure via consumption of treated products. Based on literature data from human and animal studies safe levels for daily oral exposure to the various botanical preparations and/or their active ingredients were derived and these outcomes were compared to the estimated maximal daily intake of residues of the botanical pesticides expected to be present on pepper berries treated with these preparations as pesticides. Results indicate that use of extracts of sweet flag containing beta-asarone would result in a MoE that would not indicate a high priority for risk management. However, because for beta-asarone restrictions in applications as food additive are indicated and since use as a pesticide implies an avoidable risk, it is concluded that the application of this botanical pesticide should not be encouraged. The use and use levels of the other three botanical pesticides evaluated are not of safety concern (Chapter 6). Finally, in chapter 7, the implications of the presented work are discussed, with emphasis on the potency, suitability and the safety of the botanical pesticides when used against pests associated with pepper plants. Overall, the results obtained confirm the hypothesis that botanical pesticides have the potency to be used to control pests of black pepper, providing a promising alternative for synthetic pesticide use, especially because they pose lower risks for the local environment.

Samenvatting

Zwarte peper (*Piper nigrum* L) is een belangrijk product voor Indonesië, waar het al gecultiveerd wordt sinds de zesde eeuw. Peper is vooral ook van lokaal economisch belang omdat 95% van alle peperplantages bewerkt worden door kleine boeren. Vanwege deze grote economische waarde wordt er veel aandacht besteed aan het beperken van productieverliezen door nematoden en insecten die de wortels aanvreten, in de stengel boren of de vruchten of bloemknoppen aantasten zoals *Meloidogyne incognita, Lophobaris piperis, Dasynus piperis*, en *Diconocoris hewetti*. Momenteel gebruiken boeren standaard synthetische pesticiden om deze plagen te bestrijden, en dat gebeurt over het algemeen niet volgens de voorschriften, met als gevolg dat een gezondheidsrisico voor vooral de boeren en het natuurlijke milieu ontstaat (hoofdstuk 2). Daarom is het belangrijk om een veiliger en goedkoop en eenvoudig beschikbaar alternatief te zoeken voor het gebruik van synthetische pesticiden. Een mogelijk alternatief kan gevonden worden in het gebruik van pesticiden van plantaardige oorsprong, de zogenoemde botanische pesticiden. In Indonesië worden botanische pesticiden van oudsher al gebruikt en het land is rijk aan potentieel bruikbare plantensoorten. Het zoeken naar botanische alternatieven wordt verder gestimuleerd door de toenemende vraag van consumenten naar biologisch geproduceerde producten. Voor dit onderzoek zijn zeventien kansrijke plantensoorten geselecteerd en hun ethanol extracten zijn getest op de activiteit tegen de wortelknolnematode *M. incognita* (hoofdstuk 3). Van deze zeventien plantenextracten blijken er vier actief tegen de nematode. Uit een grootschaliger experiment met hele planten in kassen, komt vooral het extract van de knoppen van *Syzygium aromaticum* L (kruidnagel) naar voren als een zeer effectief alternatief voor de aanbevolen synthetische pesticiden (hoofdstuk 3). In hoofdstuk 4 wordt de toxiciteit van de zeventien plantenextracten beschreven voor het modelinsect *Tribolium castaneum*. Hier komen als veelbelovende kandidaten de extracten van *Chrysanthemum cinerarieaefolium* (wormkruid), *Acorus calamus* L (kalmoes), *Nicotiana tabacum* L (tabak), *Syzygium aromaticum* L (kruidnagel)., *Cymbopogon citratus* DC (citroengras), *Azadirachta indica* A Juss (neem), *Andropogon zizanioides* L (khus-khusgras), *Annona muricata L)* (graviola), *Pelargonium citrosa* Van Leenii (citrosa) en zwarte peper zelf naar voren. Vervolgens (hoofdstuk 5) is er een formulering gemaakt van een combinatie van drie van de meest actieve extracten, die van wormkruid, kalmoes en kruidnagel. Deze formulering is getest in zowel laboratorium- als veldexperimenten, en naast toxiciteit blijkt de effectiviteit ook sterk het gevolg van de plaagverdrijvende werking van de ingrediënten van deze extracten tegen *L. piperis*. Veldexperimenten wijzen uit dat de formulering de druk van de meeste plaagsoorten van zwarte peper sterk kan verminderen terwijl ondertussen de giftigheid voor de natuurlijke vijanden van deze plaagsoorten veel minder is dan die van het aanbevolen synthetische bestrijdingsmiddel Deltamethrin. Uit de veldexperimenten blijkt dat binnen negen uur na toepassing de behandelde planten weer worden bevolkt door mieren en spinnen, vermoedelijk door de snelle natuurlijke afbraak van het botanische pesticide. Deze resultaten bevestigen dat de nieuwe botanische formulering een effectief en milieuvriendelijk alternatief is om plagen van zwarte peperplantages te beperken (hoofdstuk 5).

Tenslotte is een schatting gemaakt van de veiligheid voor de consument van de consumptie van zwarte peperkorrels behandeld met extracten van wormkruid, kruidnagel, kalmoes, en van het nog steeds gebruikte *Derris elliptica* Benth (derris) als pesticide (hoofdstuk 6). Gebruik makend van literatuurgegevens van studies met mensen en dieren is er een veilige dagelijkse inname bepaald en deze is vervolgens vergeleken met de geschatte maximale dagelijkse inname van mogelijke residuen van de botanische pesticiden en/of de actieve ingrediënten op de geconsumeerde pepers door de consument. Uit deze analyse blijkt dat het gebruik van extracten van kalmoes, dat het genotoxische beta-asarone bevat, leidt tot een niet verontrustende blootstelling. Maar omdat er restricties zijn voor het gebruik van beta-asarone als voedseladditief is het vermijdbare gebruik als pesticide toch af te raden. Het gebruik van de overige drie geëvalueerde botanische pesticiden geeft geen reden tot zorg. Als laatste worden in hoofdstuk 7 de implicaties van het gepresenteerde onderzoek besproken, met nadruk op de effectiviteit, veiligheid en bruikbaarheid van het gebruik van botanische pesticiden tegen plaagsoorten in peperplantages. De resultaten verkregen in dit promotieonderzoek bevestigen de vooronderstelling dat botanische pesticiden nuttig en veilig gebruikt kunnen worden voor het bestrijden van plagen in zwarte peperplantages en een veelbelovend alternatief zijn voor synthetische pesticiden.

Zwarte peper (*Piper nigrum* L) is een belangrijk product voor Indonesie, waar het al gecultiveerd wordt sinds the 6e eeuw. Peper is vooral ook van lokaal economisch belang omdat 95% van alle peper-plantages bewerkt worden door kleine boeren. Vanwege deze grote economische waarde wordt er veel aandacht besteed aan het beperken van productieverliezen door nematoden en insecten die de wortels aanvreten, in de stengel boren of de vruchten of bloemknoppen aantasten zoals *Meloidogyne incognita, Lophobaris piperis, Dasynus piperis*, en *Diconocoris hewetti*. Momenteel gebruiken boeren standaard synthetische pesticiden om deze plagen te bestrijden, en dat gebeurt over het algemeen niet volgens de voorschriften waardoor een gezondheidsrisico voor vooral de boeren en het natuurlijke milieu ontstaat (Chapter 2). Daarom is het belangrijk om een veiliger en goedkoop en eenvoudig beschikbaar alternatief te zoeken voor het gebruik synthetische pesticiden. Een mogelijk alternatief kan gevonden worden in het gebruik van pesticiden van plantaardige oorsprong, de zogenoemde botanische pesticiden. In Indonesie werden botanische pesticiden van oudsher al gebruikt en het land is rijk aan potentieel bruikbare plantensoorten. Het zoeken naar botanische alternatieven wordt verder gestimuleerd door de toenemende vraag van consumenten naar biologisch geproduceerde producten. Voor dit onderzoek zijn 17 kanrijke plantensoorten geselecteerd en hun extracten getest op de activiteit tegen de wortelknol-nematode *M. incognita* (hoofdstuk 3). Van de ** plantenextracten bleken er ** in meer of mindere mate actief tegen de nematoden, en de vormen van de gedode nematoden verschillend karakteristiek tussen groepen van pesticiden en plantenextracten wat waarschijnlijk samenhangt met het werkingsmechanisme. Uit een grootschaliger experiment met hele planten in kassen kwam het extract van de knoppen van clove naar voren als een zeer effectief alternatief voor de aanbevolen synthetische pesticiden. (hoofdstuk 3). IN hoofdstuk 4 wordt de toxiciteit van de 17 plantenextracten describes beschreven voor het model insect *Tribolium castaneum*. Hier kwamen als veelbelovende kandidaten pyrethrum, sweet flag, tobacco, clove, lemongrass, neem, vetiver, graviola, citrosa en zwarte peper zelf naar voren. Vervolgens is er een formulering gemaakt van een combinatie van 3 van de 10 meest actieve extracten. Deze formulering is getest in zowel laboratorium als veldexperimenten, en naast toxiciteit bleek de effectiviteit ook sterk het gevolg van de plaagverdrijvende werking van de ingrediënten pyrethrum, sweet flag en clove tegen *L. piperis*. Veld experimenten wezen uit dat de formulering de druk van de meeste plaagsoorten van zwarte peper sterk kan verminderen terwijl ondertussen de giftigheid voor de natuurlijke vijanden van deze plaagsoorten veel minder is dan die vanhet aanbevolen synthetische bestrijdingsmiddel deltamethrin. Uit de veldexperimenten blijkt dat binnen 9 uur na toepassing de behandelde planten weer worden bevolkt door mieren en spinnen. Vermoedelijk door de snelle natuurlijke afbraak van het botanische pesticide. Deze resultaten bevestigen dat de nieuwe botanische formulering een effectief en milieuvriendelijk alternatief is om plagen van zwarte peper plantages te beperken (Hoofdstuk 5).

De veiligheid van met pyrethrum, clove, sweet flag, of derris als pesticide behandelde zwarte pepers voor de consument is geschat op basis van menselijke orale consumptie van het behandelde product. Gebruik makend van literatuur gegevens van studies met mensen en dieren is ere en veilige dagelijkse inname bepaald en deze us vervolgens vergeleken met de geschatte maximale dagelijkse inname van residuen van de botanische pesticiden en/of de actieve ingrediënten op de geconsumeerde pepers. UIt deze analyse blijkt dat het gebruik van extracten van sweet flag dat het genotoxische beta-asarone bevat weliswaar leidt tot een niet verontrustende blootstelling (MoE), maar omdat er restricties zijn voor het gebruik van beta-asarone als voedsel-additief is het vermijdbare gebruik als pesticide toch aft e raden. Het gebruik van de overige 3 geevalueerde botanische pesticiden geeft geen reden tot zorg (Hoofdstuk 6). Als laatste worden in hoofstuk 7 de implicaties van het gepresenteerde onderzoek besproken met nadruk op de effectiviteit, veiligheid en bruikbaarheid van het gebruik van botanische pesticiden tegen plaagsoorten in pepper plantages. De resultaten verkregen in dit promotieonderzoek bevestigen de vooronderstelling dat botanische pesticiden nuttig en veilig gebruikt kunnen worden voor het bestrijden van plagen in zwarte peper plantages als veelbelovend alternatief voor synthetische pesticiden.

About the author

Wiratno was born on July 2nd 1963 in Jakarta, Indonesia. He obtained a B.Sc. degree in Plant Protection from The University of Jenderal Soedirman, Purwokerto, Indonesia on January 1988. In the same year he has been admitted as a research staff member of the Plant Protection Division of the Indonesian Spice and Medicinal Crops Research Institute (ISMCRI), which in 2005 was renamed to the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI). His main duty was conducting research in the area of crop protection strategy based on environmentally friendly approaches such as the application of botanical pesticides and of natural enemies. From 1992 until 1995 he was one of the editors of the scientific journal of the institute, and from 1995-1997 he was the Secretary of the Industrial Crops Research Program of the ISMCRI. In November 1997 he got a scholarship from the National Development and Planning Agency to get his Master's degree in Environmental Management in the New England University, Australia, which was completed in December 1998. From 1999-2001 he was the Vice Project Manager of The Integrated Pest Management for Smallholder and Estate Crop Projects (IPMSECP). In January until December 2002, he was head of the Biodiversity Division of the first Deputy of The Ministry of Research and Technology. Since July 2003 he got a scholarship from the Ministry of Agriculture to perform his PhD study in the area of Environmental Toxicology, at Wageningen University, the Netherlands.

W i r a t n o

Selected Publications

1) **Wiratno**, Taniwirjono, D., Van den Brink, P. D., Rietjens, I.M.C.M., Murk, A.J 2006. Study on the utilization of insecticides in black pepper plantation; Case s*tudy in Bangka Island, Indonesia*. Environmental Toxicology 22(4):405-414.
2) **Wiratno**, Taniwirjono, D., Rietjens, I.M.C.M., Murk, A.J, 2005. Potential, effectiveness and adverse effects of botanical pesticides used in black pepper plantations. Proceedings of the 1st International Conference of Crop Security, Brawijaya University, Malang, Indonesia. Pp. 377-386.
3) **Wiratno** and Siswanto, 2001. Study on population dynamics of *Spathius piperis*, larvae parasitoids of *L. piperis*. Proceeding of the National Seminar of Entomological Congress, Sukamandi, 13-14 March 2001.
4) **Wiratno** and E. A. Wikardi, 2001. Biodiversity of *Helopeltis* spp. in Indonesia. Presented on Symposium of Arthropods Biodiversity on Agricultural Production System. Bogor.
5) **Wiratno**, I.M. Trisawa and Siswanto, 2000. Effect of methyl eugenol to *Bactrocera dorsalis* based on the difference of diameter and number of holes. Proceeding of National Seminar for Botanical Pesticides, Vol. III, pp. 132-140.
6) I.M. Trisawa and **Wiratno**, 2000. Olfactory responses of Bactrocera dorsalis to the combination of *Melaleuca bracteata* oil and fruit extractions. Proceeding of the National Entomological Congress Vol. IV. Pp. 147-155.
7) Siswanto, I.M. Trisawa, and **Wiratno**, 1999. Utilization of virus and botanical pesticides to control *M. maculifascia*. Proceeding of The National Entomological Congress. Vol. III, pp. 126-132.
8) Siswanto, E. A. Wikardi and **Wiratno** 1999. Intercropping of Cashew with Caliandra to encourage potency of insect polinators. Technical Report of The RISMC, FY. 1999/2000
9) Siswanto, **Wiratno** and I. M. Trisawa, 1998. Identification of an insect pest attacking *Caesalpinia sappam* bean, Warta Tumbuhan Obat Indonesia. Vol. IV. No. 3.
10) Deciyanto S., I. M. Trisawa and **Wiratno**, 1998. Key pests of Patchouli oil plants and its control strategies, Patchouli oil Monograph, No. 5. Balittro, Bogor.
11) Siswanto, I.M. Trisawa, and **Wiratno**, 2000. Study on the utilization of virus and some botanical pesticides to control *M. maculifascia*. Proceeding of National Entomological Congress. Vol. III, pp. 126-132.
12) **Wiratno**, and E.A. Wikardi, 1997. Ecobiology of *Helopeltis theivora* (Miriidae; Heteroptera) on Cashew Plants (*Anacardium occidentale*). National Proceeding of Entomological Congress, Bogor, January 8th, 1997.

13) E.A. Wikardi and **Wiratno**, 1996. Key pests of Cashew and its control strategies, Proceeding of The Scientific Communication Forum of Cashew, Bogor, March 5-6th, 1996.
14) **Wiratno**, E.A. Wikardi and I. M. Trisawa, 1996. Biology of *Helopeltis antonii* on cashew plants. Journal of Spice and Medicinal Crops, Vol. V, pp. 32-42.
15) Mardiningsih T.L., and **Wiratno**, 1996. Effects of patchouli oils on the development of *S. paniceum* on coriander seeds, Proceeding of National Symposium for Medicinal Crops. Vol. 1, pp. 734-737.
16) E. A. Wikardi and **Wiratno**, 1995. Ecobiology and Management of Cashew-key-pests, Technical Reports of The RISMC, 1995/1996.
17) M. Trisawa, Ma'mun and **Wiratno**, 1995. Pests and diseases of Ylang-Ylang and attack possibilities on Agroecosystem of Singkarak Lake. Proceeding of Water Conservation Technology based on Agribusiness Programs. Singkarak, December 21-22nd, 1995.
18) Soetopo D., and **Wiratno**, 1995. Study on the existence of spider in black pepper and its roles. Bulletin Littro. Vol. III (2), pp. 31-36.
19) **Wiratno** and E. A. Wikardi, 1994. Effects of *Helopeltis* spp. attack on the growth of cashew branches. Bulletin Littro, Vol. IX No. 2.
20) **Wiratno**, 1993. Preliminary research on the effectiveness of eugenol to control *Stegobium paniceum*. Proceeding of The National Seminar for Botanical Pesticides. Vol. 1. Pp. 56-59.
21) **Wiratno**, E.A., Wikardi and M. Iskandar, 1991. Effects of Mulch originally from waste of vetivers, citronella and patchouli to control *L. piperis* on pepper plants. Selected for a yearly report of The Research Institute for Spice and Medicinal Crops, pp. 1-5, Bogor.
22) **Wiratno**, 1991. Integrated Pest Management of *Maenas maculifascia*. Proceeding of The National Entomological Congress. Vol. IV., pp. 88-105.
23) **Wiratno** and D. Soetopo (1990). Efficacy and residual effects of Endosulfan, Permethrin, and Fenitrothion insecticides for controlling *Lophobaris piperis* on pepper plants, Bulletin Littro. Vol. V, pp. 115-120.

Completed training and supervision plan

Component	Activity
A2-SENSE	Writing a research proposal
S130	Environmental Toxicology
	General Toxicology
Other PhD Education and Training Components	
Research Skills	Elaboration of the research set-up, work plan and time schedule
	Writing of the research proposal and presented on and reviewed by the Asian Development Bank
Training	Site-specific training in the use of equipments in the Sub Department of Toxicology and Laboratory of Nematology, Wageningen University
	External training and working period at a scientific (foreign) research Institute - Indonesian Centre for Agricultural Biotechnology and Genetic Resources Research and Development (April1st-May30th, 2004) - Post Harvest Division of the Indonesian Medicinal and Aromatic Crops Research Institute (November 1st, 2005 – July 23rd, 2006).
Presentations	**SENSE meeting** - Symposium of Sensible Water Technology (Leeuwarden, 12-13 April, 2007) - Summer Symposium of Human Dimensions of Global Environment. (Amsterdam, 24-26 May, 2007)
	International Symposium/ conference - The 1st International Conference of Crop Security (ICCS), (Indonesia, 20-25 September, 2005)