Traditional African plant products to protect stored cowpeas against insect damage;

THE BATTLE AGAINST THE BEETLE

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Traditional African plant products to protect stored cowpeas against insect damage;

THE BATTLE AGAINST THE BEETLE

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op dinsdag 12 november 2002
des namiddags te 13.30 uur in de Aula

Boeke, Sara J. 2002.
Traditional African plant products to protect stored cowpeas against insect damage; The battle against the beetle

Thesis Wageningen University. – with ref. – with summary in English, Dutch and French

ISBN: 90-5808-736-0

STELLINGEN

1. Goede tradities kunnen maar beter in ere gehouden worden
   Dit proefschrift; Sinterklaas

2. Laboratoriumstudies naar in het veld toe te passen methoden of technieken hebben weinig nut als de resultaten niet ook in het veld worden getoetst

3. Het schrijven van een overzichtsartikel door een beginnend promovendus is nuttig, maar kan het enthousiasme van de auteur behoorlijk temperen

4. De bewering 'het is natuurlijk, dus het kan geen kwaad' die bij het aan de man brengen van huismiddeltjes gebruikt wordt, zou in twijfel getrokken moeten worden.
   Dit proefschrift

5. Als maat voor het aantal zaden in een monster zijn volume-eenheden geschikter dan gewichts-eenheden
   Boeke J.E. en W. Kuiper, 1966. Mededelingen van het Rijksproefstation voor Zaadcontrole 17, 53-58

6. Over het 'roepen om hulp' door planten als ze aangevallen worden door insecten is het laatste woord nog niet gezegd, zeker niet als het gaat om zaden
   J.L.M. Steidle, 2000. Entomologia experimentalis et applicata 95, 185-192

7. De afkorting WUR is gezien de verengelsing van de samenleving en de wetenschap onuitsprekelijk ongelukkig gekozen

8. De verspreiding van Coca cola is succesvoller dan die van de meeste plaaginsecten

9. Soms is altijd een goed antwoord
   naar M. Kaay, lerares Frans

10. Wiskundigen zijn een soort Fransen: wat je ook tegen ze zegt, ze vertalen het meteen in hun eigen woorden en maken er zo iets totaal anders van
    naar J.W. von Goethe

11. Behalve lolbroeken, grapjassen en geinjurken is er weinig humor in het Nederlandse modebeeld

12. Blond is slechts een levensfase

13. Brutalen hebben de halve wereld, eigenwijzen hun hele

14. De keuze tussen muziek en wetenschap wordt vaak te definitief gemaakt. De twee gaan goed samen

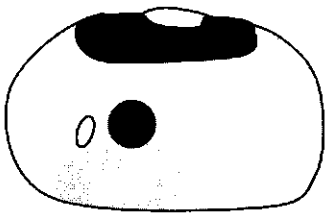
15. Het verschil tussen een bikkel en een sukkel is vaak kleiner dan twee letters
    Waarschuwing bij het strikt leven naar NJN-normen

Stellingen behorend bij het proefschrift van Sara Boeke

Traditional African plant products to protect stored cowpeas against insect damage; THE BATTLE AGAINST THE BEETLE

Wageningen, 12 november 2002

Voor hen die leren leuk maken



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Chapter 1

Plants with insecticidal properties for the protection of crops - back to basics

Non-host plant resistance

In the search for new compounds with insect-control potential, the environment itself could be a source of inspiration. The plants that are attacked by insects are not harmless. The selection of plants with anti-insect properties for example, is not difficult. Certain plants are hardly attacked by insects because they have some innate defence mechanism. This mechanism can consist of external structures such as nettle hairs or thorns, or of secondary metabolites on the surface or inside the plant that are toxic, repellent or invoke an anti-feedant effect to non-specialised insects (Schoonhoven *et al.*, 1998). The insecticidal secondary metabolites from one plant could be applied to other plants as powders or extracts and could thus protect the latter against insects.

There are disadvantages of such an approach. The plant compounds that have an anti-feedant effect on insects are usually very bitter or nasty tasting and may thus be disliked also by mammals including humans. Moreover, it should be taken into account that plants originally are source of many biologically active compounds as used for pharmaceutical purposes. The fact that insects do not attack certain plants could be due to compounds that are toxic to other organisms as well. Therefore, plant secondary metabolites should be investigated profoundly for their-side effects before being used on (food) products.

The availability of plants that are to be used as insecticides should be taken into account as well. If a plant species is used to protect a crop, it should be available in large enough quantities at the time when it is needed, that is before the insect becomes a pest. Plants or parts of plants in nature are mostly not available during the whole year since they depend on climatic seasons. Moreover, once a plant has been discovered as a potent protective agent and is used as such at larger scale, it might become rare if it is not grown purposely.

In the developed world

In developed countries, regulations for food safety and quality management are usually enforced by law to protect consumers. If products are properly treated with insecticides, the side effects of their residues on mammals are acceptably small and well known.

The use of pesticides is subject to dynamic trends. Insects might develop resistance against single component pesticides (Ayad and Alyousef, 1986; Evans, 1985), or the residual and side effects of the agent on consumers' health and on the environment may

prove unacceptable. For these and other reasons, compounds that are used successfully now might be prohibited in the near future, as was for example the case with most chlorinated pesticides (such as DDT). Plants could then provide leads in the search for new insecticides.
The concentration of secondary compounds in plants is often low or very low, differs with the plant part and changes with the plant age, growing situation etc. (Schoonhoven *et al.*, 1998). Extraction and concentration of the active compounds will often result in more effective products, since the ineffective bulk of primary and secondary plant compounds is removed and only the required compounds are retained. In the developed world, money and equipment are usually available to identify, extract, or even to synthesise the pure insecticidal compounds from plants. These active compounds can then be used in quantified mixtures as insecticides. Investigations concerning toxicity and effects on organisms are almost exclusively done on such pure compounds. When dealing with biologically variable mixtures such as complete plants or extracts this becomes technically much more difficult and very expensive.

In developing countries
In tropical countries, plants have been and are still used as protective agents or insecticides. However, with the introduction of often subsidised chemical insecticides, much of this traditional knowledge was lost (Atteh, 1984). With the introduction of structural adjustment programmes, the subsidies stopped and the synthetic chemicals were no longer affordable for most of the low-income producers. Growers would still want to treat their crops in the field and after harvest to protect them until they are needed for home consumption or for selling. However, nowadays much damage occurs in the untreated fields already and most of the material is sold immediately after harvest for a low price because proliferating insects will make the products worthless within a short period. The yield and the price the growers receive could be much higher if they were able to protect their produce from insects during several months.
If the traditional knowledge could somehow be restored, the protection of crops with plant materials could become general practice again. The technology, money, means and the legal need to look for the active ingredients may not be present, but the actual sources of insecticidal compounds, the plants, are available.
Advantages of the application of nearby growing plants as insecticides are that such plants could offer a cheap alternative for synthetic insecticides. They would also be relatively easy to obtain and application normally should not bring about serious health risks for the person handling them. Many plants have been used traditionally for many generations. Therefore, the toxicity to human consumers of the treated products is likely to be acceptably low. The method can be looked upon as environmentally sound, since

no new residues are brought into the environment. An additional advantage would be that the development of resistance would take longer if a mixture of compounds is used instead of one purified compound.

Cowpea and its main storage insect pest

In this thesis, a specific problem will be tackled: that of the cowpea (*Vigna unguiculata* (L.) Walp.) and its main storage insect pest. Cowpea is an important crop in tropical regions, particularly in West Africa. The seeds are rich in protein and B-vitamins (Phillips and McWatters, 1991) and are therefore important in the diet of many low-resource subsistence farmers as 'the meat of the poor'.

In the field, apart from various other pests, several seed beetle species lay their eggs on the surface of maturing pods or on ripening seeds. The most important species is the cowpea beetle, *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae). This beetle is responsible for over 90% of all insect damage to cowpea seeds (Caswell, 1981). The larvae develop inside the bean, destroying its contents and after a few weeks, new adults emerge ready to mate and oviposit on the available beans. With the harvested beans, beetles and their eggs are taken from the cowpea field to the storage room where infestation may reach 100 % within 3 to 5 months (Singh, 1977). Due to this insect's damage, the germination of the seeds is negatively affected, and fungal infection occurs more often (Charjan and Tarar, 1994).

In the field

There are many environmentally sound ways to keep insect infestation in stored cowpea seeds at low levels (see Van Huis, 1991). The first step should be to keep the infestation level of freshly harvested beans as low as possible.

Growing resistant varieties, if they are available, is one option. However, in West Africa, availability of such varieties is often limited and the durability of such resistance might be questioned (Kitch *et al.*, 1991). Besides, with the introduced resistance against bruchids, other properties of the cowpea plant might be changed (Jackai and Ng, 2001), causing other constraints for obtaining good bean harvests (Singh *et al.*, 1992). Moreover, new bean varieties could have different seed colours, textures, sizes, and tastes, and would therefore be potentially unacceptable as food or unsuitable for cultural events such as religious ceremonies.

Inter-cropping could help to lower the incidence of cowpea pest insects and thus prevent infestation of the seeds. A crop growing in the same field could serve as a refuge or a source of food for parasitoids and predatory insects (Khan *et al.*, 1997). Weeding the

field to remove possible alternative host plants is another measure that could be taken to keep initial infestation at a low level.
Altering harvest time, getting (part of) the crop off the field before the ripe seeds attract insects could also prevent severe infestation. If the beans are then stored in the pod in clean storage structures, the infestation rate can be kept as low as possible (Van Huis, 1991).

From the field to storage

At harvest, infestation will still be present but in low numbers. Prevett (1961) estimated that at least two percent of the beans will show traces of beetle infestation. Some of the infested beans could be picked out, but it is very difficult to remove all infested beans. Therefore, control measures should be focussed on prevention of further development of a beetle population.
Proper drying of the beans before they are put into store lowers the beetles' reproductive success (El-Sawaf, 1956). Moreover, dry seeds are less susceptible to mould and fungi that often come with beetle infestation.
Raising the temperature in the storage room to at least 47 °C for more than four hours kills the adult beetles present between the beans (Iloba and Osuji, 1986). If the temperature reaches over 57 °C for at least one hour, all developmental stages of the beetle are killed (Kitch *et al.*, 1992). In tropical countries, these temperatures could be reached if the beans are either hung over a fire or exposed to the midday sun in plastic bags or on a black sheet of plastic covered by a transparent one (Chinwada and Giga, 1996). If plastic is available, and if the treatment is repeated when needed, this could be a good method to reduce infestation.
Storing beans in airtight structures, bottles, plastic bags, oil drums etc. that are filled to the rim with seeds would cause the developing insects to use up all the oxygen within two weeks and to suffocate before they can do serious damage (Caswell, 1973).
The beans could also be disinfested by freezing them, irradiating them with gamma rays (Ghogomu, 1990), or keeping the stored beans under a controlled atmosphere containing a high level of carbon dioxide (Mbata and Reichmuth, 1996) or nitrogen concentrations (Ofuya and Reichmuth, 1994). However, for most subsistence farmers these methods, due to a lack of financial resources, material and equipment, are not applicable.

In storage

When the beans are stored, they could be treated in different ways for protection against insects. The most obvious way seems to be the treatment or fumigation with synthetic pesticides. Many of these chemicals have proven to be very effective against bruchid

damage when applied at the right time, at the right dose and using appropriate techniques and material for the application. For low-income families in villages, however, these chemicals are often not available and the costs are disproportionately high whereas a lack of knowledge about the application may reduce the efficacy of the pesticide and can cause hazardous situations for appliers and for consumers of the beans. Improper use of pesticides, measured over two months only in a part of Benin, led to 24 fatal accidents and 241 cases of acute poisoning (Tovignan *et al.*, 2001). Apart from the possible development of resistance in the beetle, a major disadvantage is that these insecticides kill all insects, including beneficial ones such as the natural enemies of the beetles.

If the beans are left untreated, many of the developing beetles will be parasitised by specialised parasitic wasps (Caswell, 1973). Parasitisation by different parasitoid species (see Van Huis, 1991) can occur in all developmental stages of the beetle: as eggs (by *Uscana* spp.), larvae or pupae (by *Dinarmus basalis* or *Eupelmus vuiletii*). In the laboratory, under optimised conditions, parasitisation can cause the death of up to 82% of the developing beetles (Cortesero *et al.*, 1997). In the field and in untreated stores, the parasitoids can suppress the build-up of beetle populations, but the control is never 100%.

As an alternative for synthetic pesticides, fine sand or ash can be mixed with stored beans to make a physical barrier which prevents emerged adult beetles from finding each other for mating or from reaching a next bean to oviposit on. These particulate materials could interfere with the respiratory ability of adults, larvae and possibly eggs, or cause abrasions to the eggs and adults on the bean surface (Katanga Apuuli and Villet, 1996). The large quantities of the protective material needed make this method of protection less practical, especially for considerable quantities of stored beans.

In traditional practice, plants are used to treat stored products. These could be applied in many different forms: as whole plants in layers between pods or seeds, as powders, extracts or oils mixed with seeds or as volatile oils or extracts acting as fumigants (Boeke *et al.*, 2001). Such insecticidal plant products, applied to stored beans, can effectively protect stored cowpeas against the progress of bruchid infestation (Boeke *et al.*, 2001).

Examples: the neem tree and tephrosia

Very few insects feed on the neem tree, *Azadirachta indica* Juss. (Meliaceae). This tree grows everywhere in the tropics. It does not have external defence structures, but it contains among others a group of compounds named limonoids, of which azadirachtin is the best known representative (Van der Nat *et al.*, 1991). This compound has a strong antifeedant effect on all kinds of insects and it affects oviposition behaviour,

metamorphosis and fecundity of insects. The highest yield of azadirachtin reported is 10 g/kg from the kernels of the fruits (Schmutterer, 1990). The oil pressed from these kernels, when applied to cowpea seeds completely inhibits the development of a bruchid beetle population. Normal numbers of eggs are laid, but they do not hatch and no emerging adults are found (see chapter 4).

The effective compound is not (very) toxic to humans (Beard, 1989) or other mammals (see chapter 6). The use of azadirachtin in mixtures is allowed in some countries of the developed world (e.g. Azatin: USA Environmental Protection Agency, Registration number 62552-1), whereas the use of crude neem oil is prohibited in most developed countries. For the use of neem derivatives in the developing world, some state that they could easily, safely and effectively be applied to stored seeds (Anonymous, 1995; Saxena *et al.*, 1989). Oil from neem kernels can be easily extracted, even by hand. The kernels are present wherever the neem tree is and they are mainly used for the extraction of the oil. Others would say that due to its very bitter taste (Lale and Mustapha, 2000), and the possibility of aflatoxins in the neem seeds due to fungal infection, the use of neem oil on stored seeds for consumption should be advised against.

A less disputed insecticidal plant is *Tephrosia vogelii* Leguminosae, which has insecticidal properties and is well-known as a fish poison (Ibrahim *et al.*, 2000). All parts of this plant contain rotenone (Delobel and Malonga, 1987). This compound is insoluble in water (Brown, 1951) and in insects it acts as a respiration inhibitor (Benner, 1993) or rather as a contact poison with no fumigant effect. In insects, rotenone is a muscular depressant, which may induce slight neurotoxic symptoms; it slowly paralyses the insects due to complete muscle relaxation (Brown, 1951). The compound degrades in sunlight (Jones *et al.*, 1933) and has a very low toxicity for warm-blooded animals (Bowers, 1983).

This thesis

Plants or plant products could be used as insecticides to protect stored cowpea as is documented in part I of this thesis (Boeke *et al.*, 2001). If money allows it, or if legislation or safety aspects oblige it, the pure active compounds could be used. In other situations, the complete plant or easily obtained extracts could offer a solution for the problems of availability, health risks, costs, and resistance against synthetic pesticides. Especially resource-poor farmers in the tropics would benefit from cheap ways to protect their stored seeds.

In this second part of the thesis, the results of the search for botanical insecticides is presented as it was undertaken with and for cowpea growers in Benin, West Africa. First, the best testing system, the most susceptible bean variety and the most successful beetle strain were selected (chapter 2). The plant species that are traditionally most often

used by farmers in Benin were selected based on a questionnaire among cowpea growers. These were collected and powders of the dry plant parts were investigated for their efficacy against the cowpea beetle (chapter 3). Those powders that proved effective in the laboratory, through toxicity or repellence, were extracted with boiling water and these extracts were used in bio-assays to find out if the efficacy could thus be enhanced (chapter 4). For the most promising plant products, the effects on natural enemies of the beetles were then investigated (chapter 5). The results are presented of an experiment in the storage situation in Benin with five of the plant species as used by farmers (chapter 7). For the most famous example of botanical insecticides, the neem tree *Azadirachta indica*, an overview of the literature on its effects on mammals is given to enable evaluation of its toxicity of the treated beans to human consumers (chapter 6). In chapter 8, the results are summarised and discussed in the perspective of the possible application of the plant products in Benin. The overall aim of the project was to come up with a safe, effective plant, or more than one, to be used for the protection of cowpea in West Africa. Farmers could then store their harvest, use it for their own needs and sell the surplus for a good price.

Chapter 2

Host preference of *Callosobruchus maculatus*: a comparison of life history characteristics for three strains of beetles on two varieties of cowpea

Sara J. Boeke, Joop J.A. van Loon

Abstract

The reproductive success of *Callosobruchus maculatus* Fabricius, the main insect pest of stored cowpea, may vary between strains of this beetle and between varieties of the host seeds. Life history parameters of beetle strains from three different origins in West Africa were compared on two susceptible varieties of cowpea, *Vigna unguiculata* (L.) Walp. All beetle strains were assayed in a no-choice and a two-choice test. No major differences were found between the beetle strains. In a no-choice situation, the developmental period from egg to adult was prolonged on the bean variety Kpodjiguegue. In a two-choice situation, the beetles showed a strong preference for the Californian blackeyed bean variety to oviposit on. Here again the development took longer on Kpodjiguegue beans and the intrinsic rate of increase of the beetle population was lower. Using either equal numbers of beans of the same size or equal weights of beans of undetermined size of the two bean varieties did not affect the outcome of the test.

Key words: *Callosobruchus*, life history, cowpea, varietal difference, geographical strains

Introduction

Cowpea, *Vigna unguiculata* (L.) Walp. Leguminosae Papilionoideae is an important source of protein in the diet of many people in tropical areas. In the field, the crop is victim of many pests and diseases, whereas in seed storage, the main problem, apart from moulds and rodent damage is caused by only one insect species, the cowpea weevil *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae). This bruchid beetle lays its eggs on the ripe pods or seeds. The larvae feed on the contents of the seed and emerge from the hollowed bean as adults, ready to mate and lay eggs. The generation time of the beetle is about three weeks, but varies depending on the temperature and the humidity of the seeds and their environment (El-Sawaf, 1956; Mookherjee and Chawla, 1964) and on the bean variety (Credland, 1987).

Callosobruchus maculatus is known to infest all grains of a complete cowpea harvest within 3 to 5 months of storage (Singh, 1977) and it is responsible for over 90 % of all insect damage to cowpea seeds (Caswell, 1981). After emergence of the beetle, seed germination and seedling vigour are negatively affected (Baier and Webster, 1992). The damaged seeds lose weight, are unacceptable for human consumption and thereby represent a lower market value (Javaid and Poswal, 1995; Elhag, 2000).

Not all of the 7000 cowpea cultivars (Singh, 1977) are equally susceptible to this pest insect. The host on which a *C. maculatus* female lays her eggs can have a great influence on the developmental rate and on the numbers of offspring the beetle eventually produces (Credland, 1987). Generally, the beetle prefers seed types with a smooth testa over rougher ones to oviposit on. A smooth testa allows for better attachment of the eggs to the seed, resulting in a higher chance of successful development (Nwanze and Horber, 1976). Most eggs are laid on the cheek of the bean (Nwanze *et al.*, 1975). The seed height and the corresponding curve of the cheek, which is preferred as flat as possible, account for much of the variance in ovipositional preference (Oigiangbe and Onigbinde, 1996). Colour preference is ambiguous: in a choice situation, darker coloured seeds are preferred for oviposition to white seeds (Chavan *et al.*, 1997), but in no choice situations, no difference was found (Shazali, 1990). Resistance of certain cultivars does not seem to be dependent on the levels of cysteine proteinase inhibitors or on tannin content (Shazali, 1990; Fernandes *et al.*, 1993).

In our laboratory, the cowpea beetles are routinely reared on the Californian blackeyed cowpea variety, which is susceptible to *C. maculatus* (Nwanze and Horber, 1975; Baker *et al.*, 1989). However, in the field situation in Benin, West Africa, the widespread cowpea variety Kpodjiguegue is highly favoured by the cowpea beetle (Kossou, pers. comm.). The seeds of this cowpea variety have a darker smoother testa than the Californian blackeyed beans, which could attribute to their attractiveness. However, there is an obvious difference in size between the two varieties. Kpodjiguegue beans are much smaller than Californian blackeyed beans, which would invoke an ovipositional preference for the bigger seeds in a choice situation (Hu *et al.*, 1995; Ofuya, 1997a).

Besides a preference of *C. maculatus* for a certain host type, there are differences in the developmental characteristics of different beetle populations (Credland *et al.*, 1986; Credland and Wright, 1989; Giga *et al.*, 1995). The host preference, numbers of offspring and the developmental period can vary considerably between separately evolved populations (Dick and Credland, 1984).

Here we test if there are differences in preference or survival on the two bean varieties for beetles collected in Niger near Niamey, in Southern Benin and in Northern Benin. The beetles from these different sites will be called beetle strains, although we do not know if they are genetically different. These three beetle strains had been reared in the laboratory for different periods of time. We tested whether the strains differ in host preference and life

history characteristics and whether they are affected differently by the rearing procedures. Thus the biology and performances of three beetle strains on two bean varieties were followed and presented here. The study serves to provide baseline data on the most successful beetle strain on the most susceptible bean variety for later evaluation of the effect of measures taken to protect stored cowpea.

Material and methods

The experiments were done in October 2000 with three beetle strains that are currently reared in our laboratory. The North Benin strain was collected in Northern Benin and reared in the laboratory since January 1999, the South Benin strain collected in Southern Benin near Cotonou was reared in the laboratory since October 1998 and the Niger strain was collected near Niamey and reared in the laboratory since December 1990.

All beetles were reared in petri dishes on the cowpea variety Californian blackeyed in separate climate chambers at 30°C ± 2°C with a twelve-hour photoperiod at the ambient relative humidity (50-80%). Tests and incubations were done under the same conditions. For the tests newly emerged (1-1.5 h), unmated beetles were used.

The beans used for the rearings and the experiments were free of insecticides. Kpodjiguegue beans were produced in Benin and transported soon after harvest. The Californian blackeyed beans were purchased in the Netherlands. All beans were disinfested in our laboratory by storing them at –20°C for one week and drying them afterwards in an oven at 60°C for one week. Before use, they were stored in plastic containers at room temperature. For the rearing and for the experiments, visually uninfested cowpeas were used (without eggs or emergence holes).

No-choice test

For each cowpea variety, 50 beans of undetermined size and weight were put in a petri dish of 5-cm diameter. One female and two male beetles were released on these beans.

The petri dishes were monitored every 24 hours. Beans with eggs were replaced by uninfested ones once a day until the death of the adult beetles. For every day for each female, the beans with eggs were stored in separate petri dishes under the incubation conditions. In this way, the daily egg production, the lifetime-fecundity and adult longevity in days were measured for the individual beetles.

The eggs were incubated to monitor daily emergence, to determine the developmental period and egg and larval mortality. Newly emerged adults were sexed and removed daily. The first emerging F_1 adults were used to start the F_2 experiment, which was treated and monitored in the same way as the F_1 generation.

The intrinsic rate of increase of the beetle population per day, r_m was calculated according to Howe's (1953) simplified method: $r_m = \ln x / (t + 0.5*p)$ with x = the number of female

offspring that emerged, t = the developmental period in days and p = the longevity of the female parent beetle (= the oviposition period). The experimental scheme is shown in Figure 1. The tests were replicated five times

Two choice test

When compared directly, the difference in average size between the bean varieties could be an important factor in the beetles' decision to oviposit. To eliminate the effect of this size factor, we did two experiments:

-Equal numbers of beans: Of each variety, 25 beans of approximately equal size were put in one 5-cm petri dish. For the Californian blackeyed variety, the smallest beans were selected and for Kpodjiguegue, the biggest seeds were used. One newly emerged female and two newly emerged males were added to the beans. Data collection was the same as in the first experiment. Only one generation was incubated.

-Equal weight of beans: For each bean variety five grams of beans of undetermined size were weighed in a 5-cm petri dish and infested, incubated and monitored as in the former experiment. These tests were replicated five times

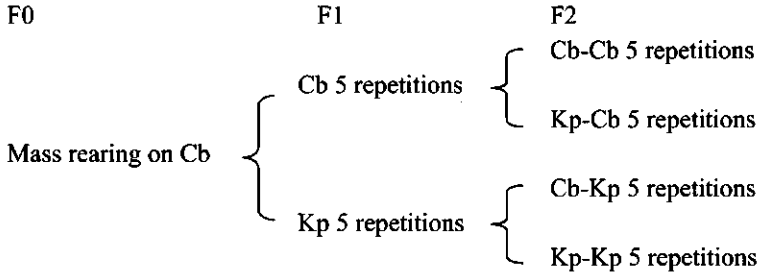

Figure 1: Scheme of the treatments in the no-choice test as they were tested for three strains of the cowpea beetle Callosobruchus maculatus. Cb = Californian blackeyed beans, Kp = Kpodjiguegue beans. E.g. Cb-Kp = parents reared on Kp, actual data collected from offspring living on Cb beans.

Statistics

The data were analysed with a Multivariate General Linear Model with the beetle strain, the bean variety of the current generation and the bean variety on which the parents developed as fixed factors. A Bonferroni post-hoc test was performed. For the no-choice test, the data for the two subsequent beetle generations were analysed separately.

Results

No-choice test

The beetle's generations will be referred to as shown in Figure 1. The results for oviposition and adult longevity and the data on the development of the F_1 and F_2 generations of the no-choice test are shown in Table 1.

Among the beetle strains, no differences were observed for the F_0 and the F_2 generation. The only difference that could be found in the F_1 generation was that male beetles of the strain from Southern Benin lived longer than those of the Niger strain. The strain from North Benin did not show differences from the other strains.

The bean variety offered to oviposit on had an effect on the longevity of male beetles of all strains. These lived longer on Kpodjiguegue beans for the F_0 generation as well as the F_1 generation.

The bean variety in which the beetles developed affected the developmental period. The development took longer on Kpodjiguegue for all beetle strains and for both generations. The number of emerging beetles and the percentage of eggs that did not complete the development were not different. The intrinsic rate of multiplication of the beetle population (r_m) was lower on Kpodjiguegue beans for all beetle strains due to the longer developmental period.

The bean variety on which the F_1 generation had been reared had an effect on the number of eggs laid after 24 hours. Beetles of all strains that had emerged from Kpodjiguegue laid more eggs in the first twenty-four hours of their lives irrespective of the bean they were offered to oviposit on. There were no differences between bean varieties for the total lifetime fecundity.

The bean variety on which the previous (F_0 or F_1) generation developed had no influence on the development of the next generation (F_1 or F_2).

Table 1: Life history values for the F_0, the F_1 and the F_2 generation of three strains of cowpea beetles on two cowpea varieties. Values represent means of five replications ± standard deviation. Abbreviations for the bean varieties are explained in Figure 1. Values in columns followed by the same letters are not significantly different ($P>0.05$). Capital letters indicate the influence of the beetle strain, small letters that of the bean variety on which the present generation lives, and Greek letters that of the bean variety on which the previous generation had developed.

Bean variety	Beetle strain*	Number of eggs Day 1	Total	Longevity Female	Male	Developmental period (days)	Number of beetle emerged	Percentage mortality	r_m * 100
F_0						F_1			
Cb	North	33.2 ± 8.5 Aa	92.6 ± 21.7 Aa	7.6 ± 2.5 Aa	8.4 ± 0.5 Aa	22.5 ± 0.4 Aa	79.4 ± 20.8 Aa	14.6 ± 4.2 Aa	14.0 ± 1.11 Aa
Cb	South	32.8 ± 9.2 Aa	96.6 ± 20.0 Aa	7.2 ± 0.8 Aa	8.2 ± 0.8 Aa	22.6 ± 0.6 Aa	78.2 ± 19.0 Aa	19.4 ± 5.1 Aa	13.6 ± 1.01 Aa
Cb	Niger	24.6 ± 3.8 Aa	105.4 ± 19.0 Aa	8.6 ± 1.1 Aa	8.4 ± 0.9 Aa	21.6 ± 0.3 Aa	91.8 ± 22.2 Aa	13.5 ± 6.8 Aa	14.3 ± 0.97 Aa
Kp	North	30.6 ± 8.6 Aa	91.4 ± 23.6 Aa	9.2 ± 2.2 Aa	10.2 ± 0.8 Ab	25.1 ± 1.9 Ab	69.8 ± 35.0 Aa	27.2 ± 27.4 Aa	11.6 ± 2.39 Ab
Kp	South	30.6 ± 5.9 Aa	83.6 ± 35.8 Aa	7.0 ± 1.6 Aa	10.2 ± 0.8 Ab	23.5 ± 0.4 Ab	62.4 ± 32.2 Aa	27.8 ± 9.3 Aa	11.8 ± 2.30 Ab
Kp	Niger	28.8 ± 5.9 Aa	87.2 ± 14.4 Aa	9.2 ± 2.6 Aa	10.4 ± 0.9 Ab	24.1 ± 0.7 Ab	70.6 ± 13.3 Aa	19.0 ± 7.4 Aa	12.4 ± 1.23 Ab
F_1						F_2			
Cb-Cb	North	21.3 ± 5.2 Aaα	87.8 ± 18.0 Aaα	7.0 ± 2.2 Aaα	7.8 ± 0.5 ABaα	23.6 ± 0.2 Aaα	70.0 ± 12.0 Aaα	19.7 ± 5.2 Aaα	13.2 ± 0.47 Aaα
Cb-Cb	South	27.5 ± 6.0 Aaα	99.5 ± 4.9 Aaα	7.5 ± 1.3 Aaα	8.3 ± 0.5 Baα	23.0 ± 0.3 Aaα	89.5 ± 4.9 Aaα	10.0 ± 4.9 Aaα	14.4 ± 0.31 Aaα
Cb-Cb	Niger	15.5 ± 4.7 Aaα	81.3 ± 15.9 Aaα	8.5 ± 1.3 Aaα	7.8 ± 1.0 Aaα	22.6 ± 0.5 Aaα	69.3 ± 16.5 Aaα	14.8 ± 9.6 Aaα	12.9 ± 1.00 Aaα
Cb-Kp	North	22.5 ± 7.9 Aaβ	86.8 ± 12.7 Aaα	7.5 ± 1.0 Aaα	7.5 ± 0.6 ABaβ	23.5 ± 0.3 Aaα	75.3 ± 9.7 Aaα	12.9 ± 7.0 Aaα	13.1 ± 0.51 Aaα
Cb-Kp	South	36.8 ± 1.5 Aaβ	89.5 ± 5.5 Aaα	7.8 ± 1.0 Aaα	8.0 ± 1.2 Baβ	22.9 ± 0.2 Aaα	76.3 ± 3.5 Aaα	14.5 ± 6.9 Aaα	13.5 ± 0.39 Aaα
Cb-Kp	Niger	32.8 ± 7.8 Aaβ	77.3 ± 9.7 Aaα	7.3 ± 1.3 Aaα	6.5 ± 0.6 Aaβ	22.7 ± 0.4 Aaα	65.8 ± 8.2 Aaα	14.8 ± 2.5 Aaα	13.4 ± 0.78 Aaα
Kp-Cb	North	26.7 ± 5.0 Aaα	98.7 ± 17.7 Aaα	8.7 ± 1.5 Aaα	9.0 ± 1.0 ABbα	23.7 ± 0.3 Abα	85.0 ± 15.6 Aaα	13.9 ± 1.5 Aaα	13.4 ± 0.53 Abα
Kp-Cb	South	17.8 ± 8.7 Aaα	57.8 ± 29.6 Aaα	8.2 ± 2.2 Aaα	9.8 ± 1.0 Bbα	24.0 ± 0.6 Abα	48.8 ± 23.9 Aaα	14.3 ± 3.7 Aaα	10.2 ± 1.77 Abα
Kp-Cb	Niger	18.0 ± 3.5 Aaα	93.6 ± 11.2 Aaα	8.8 ± 1.3 Aaα	9.0 ± 0.7 Abα	24.7 ± 0.5 Abα	72.0 ± 15.1 Aaα	23.6 ± 10.9 Aaα	11.8 ± 1.00 Abα
Kp-Kp	North	22.8 ± 5.1 Aaβ	91.8 ± 10.4 Aaα	6.8 ± 0.4 Aaα	8.8 ± 0.8 Abbβ	24.8 ± 0.5 Abα	78.0 ± 11.2 Aaα	15.1 ± 7.1 Aaα	12.6 ± 0.80 Abα
Kp-Kp	South	28.3 ± 19.4 Aaβ	71.3 ± 48.7 Aaα	7.5 ± 3.0 Aaα	9.5 ± 1.7 Bbβ	24.2 ± 0.4 Abα	60.0 ± 41.3 Aaα	36.5 ± 42.3 Aaα	12.7 ± 0.70 Abα
Kp-Kp	Niger	18.8 ± 14.1 Aaβ	70.0 ± 39.1 Aaα	8.8 ± 1.3 Aaα	8.2 ± 0.8 Abβ	24.4 ± 0.8 Abα	55.6 ± 31.0 Aaα	16.7 ± 9.7 Aaα	9.7 ± 5.48 Abα

*North = beetle strain from northern Benin, South = beetle strain from southern Benin

Two-choice tests

The results of both two-choice tests are shown in Table 2. When given the choice, beetles of all strains prefer the Californian blackeyed beans to oviposit on. There was no difference between the two types of choice tests. The size of the beans being comparable in one of the tests did not make the beetle's preference for the Californian blackeyed bean less profound.

The beetle strains differed in the number of eggs that were laid during the first 24 hours. The Niger strain produced fewer eggs than the other two strains in the beginning of the oviposition period. This difference was not found in the total number of eggs produced by the beetles.

During the whole period of oviposition, beetles of all strains laid fewer eggs on beans of the Kpodjiguegue variety than on Californian blackeyed beans. The total number of eggs and consequently the number of emerging beetles were lower on Kpodjiguegue beans. The emergence from Kpodjiguegue was on average later. Consequently, due to fewer beetles emerging after a longer period, the r_m value was lower for Kpodjiguegue beans. For the test with equal weights of beans, the developmental period was longer for beetles of the North Benin strain than for the Niger strain. The south Benin strain did not differ from the other two strains and there was no effect on the r_m value for any of the strains. The percentage of eggs failing to develop into adults on Kpodjiguegue beans was not different for Californian blackeyed beans.

Discussion

From these tests, it appears that the beetle strains as they are reared in our laboratory do not differ much in their behaviour and biology. The developmental success and the period needed to complete the developmental cycle are comparable. These results are not surprising since the places of origin of the strains are not very far apart and the beetles were all collected on cowpea. Strains tested by Dick and Credland (1984) originated from Yemen, Nigeria and Brazil and the first strain was even collected on lentils. These authors did find differences in

the numbers of eggs laid when a certain number of cowpea beans was offered. However, when clean beans were offered daily, as in our experiment, these differences were no longer found. The developmental time was different for their strains, with the Yemen strain that had to change from lentil to cowpea as a host showing the longest developmental period.

One can never be sure that the cowpea, from which our beetle strains were collected, was grown in the region where we bought it. However, the small-scale agricultural system in West Africa and the fact that the cowpea was infested at the time of purchase makes it likely that local producers were involved in selling and thus that the beetles collected in an area did also originate from there.

Table 2: Life history values for three strains of cowpea beetles having the choice between two cowpea varieties. For the two bean varieties, either equal numbers of beans of the same size or equal weights of beans of undetermined size were provided. Values represent means of five replications ± standard deviation. Cb = Californian blackeyed beans, Kp = Kpodjiguegue beans. Values in columns followed by the same letters are not significantly different (P>0.05). Capital letters indicate the influence of the beetle strain measured over the whole replication unit, small letters that of the bean variety within the unit.

Beetle strain	Bean variety	Number of eggs Day 1	Number of eggs Total	Emergence (days after oviposition)	Number F1 emerged	Percentage mortality	r_m *100
Equal number of beans per variety							
North Benin	Cb + Kp	25.2 ± 12.0 A	72.6 ± 24.6 A	22.7 ± 0.8 A	60.2 ± 21.8 A	11.9 ± 8.0 A	11.9 ± 1.34 A
	Cb	21.8 ± 10.9 a	65.0 ± 20.0 a	22.8 ± 0.4 a	56.4 ± 14.6 a	12.3 ± 7.7 a	16.2 ± 1.01 a
	Kp	3.4 ± 2.9 b	7.6 ± 5.9 b	22.6 ± 1.2 b	6.6 ± 4.3 b	6.3 ± 14.0 a	7.1 ± 4.43 b
South Benin	Cb + Kp	29.0 ± 12.6 A	74.2 ± 17.7 A	23.3 ± 0.5 A	64.6 ± 15.5 A	12.8 ± 5.9 A	13.2 ± 0.93 A
	Cb	25.4 ± 10.0 a	67.0 ± 15.6 a	22.9 ± 0.5 a	58.8 ± 13.3 a	11.9 ± 5.1 a	14.4 ± 0.69 a
	Kp	3.6 ± 3.2 b	7.2 ± 4.3 b	23.8 ± 0.7 b	5.6 ± 3.9 b	20.7 ± 21.7 a	4.69 ± 2.62 b
Niger	Cb + Kp	13.2 ± 14.5 B	72.4 ± 15.4 A	22.8 ± 0.4 A	61.6 ± 13.8 A	14.7 ± 3.7 A	12.7 ± 0.89 A
	Cb	12.2 ± 13.6 a	68.6 ± 12.4 a	22.4 ± 0.4 a	59.6 ± 11.7 a	13.3 ± 2.7 a	15.9 ± 1.03 a
	Kp	1.0 ± 1.2 b	3.8 ± 3.6 b	23.4 ± 0.4 b	2.2 ± 2.3 b	66.3 ± 32.7 a	4.8 ± 2.21 b
Equal weight of beans per variety							
North Benin	Cb + Kp	25.8 ± 12.9 A	76.4 ± 13.4 A	23.2 ± 0.7 A	65.8 ± 13.0 A	13.3 ± 4.4 A	12.8 ± 0.78 A
	Cb	22.0 ± 9.4 a	68.4 ± 16.6 a	22.8 ± 0.4 a	59.8 ± 16.0 a	13.0 ± 5.0 a	15.4 ± 1.45 a
	Kp	3.8 ± 3.6 b	8.0 ± 4.7 b	23.6 ± 1.1 b	6.8 ± 3.8 b	12.6 ± 14.5 a	6.8 ± 1.75 b
South Benin	Cb + Kp	21.0 ± 6.0 A	59.2 ± 22.3 A	22.7 ± 0.4 AB	47.4 ± 16.1 A	19.7 ± 7.4 A	12.0 ± 1.34 A
	Cb	16.0 ± 7.1 a	48.4 ± 19.2 a	22.5 ± 0.2 a	39.2 ± 13.4 a	17.2 ± 6.4 a	13.2 ± 1.36 a
	Kp	5.0 ± 1.2 b	10.8 ± 3.3 b	22.9 ± 0.7 b	7.6 ± 4.5 b	31.3 ± 24.4 a	6.6 ± 2.41 b
Niger	Cb + Kp	10.6 ± 3.8 B	77.4 ± 17.1 A	22.4 ± 0.4 B	65.6 ± 19.3 A	15.9 ± 8.0 A	13.1 ± 1.68 A
	Cb	9.0 ± 4.2 a	71.2 ± 14.7 a	22.0 ± 0.4 a	61.4 ± 16.8 a	14.9 ± 7.1 a	16.1 ± 1.10 a
	Kp	1.6 ± 0.9 b	6.2 ± 4.2 b	22.7 ± 0.6 b	4.6 ± 3.3 b	22.2 ± 23.9 a	5.3 ± 1.98 b

Beans were handled daily during the experiments and eggs were counted within a day after oviposition. These procedures may have had an effect on the number of offspring produced. The eggs had not hatched at the time of handling so they were damaged, the embryo inside would have died due to the experimental set-up (Nwanze and Horber, 1975) and not due to unsuitability of the host for that particular beetle strain. However, the procedures were the same for all treatments during the whole test period, so the damaging effect is supposed to be equally severe for all eggs on all beans. If there was any effect of these procedures on the outcome of the tests at all, it would have been advantageous for eggs on Kpodjiguegue beans, since the eggs were better visible on the dark testa of this variety.

In the two-choice tests, we could not distinguish the beetles emerging from the two bean varieties. Since the two bean varieties bearing eggs were incubated together in one petri dish, the only proof of the number of beetles emerged from one variety was the hole they left in the bean. The gender of the beetles emerging from the beans could only be determined for the daily total of emerged beans, not for the separate bean varieties.

The size of the beetles used to infest the experimental units could influence their performance. Heavier beetles live longer and have a higher fertility than lighter beetles (Wilson, 1988). However, for the infestation of the experimental units, beans from the mass-rearing containing beetles ready to emerge were kept separately until enough beetles had emerged to infest all units. Infestation took place in random order. Thus, we presume that the differences in beetle longevity on different bean varieties were not dependent on the differences in size or age of the beetles at the time of infestation.

Emergence as smaller adults which have a shorter life span and produce fewer offspring (Wilson, 1988) is one of the effects on larvae developing on a less suitable host. However, these larvae could compensate for this drop in fitness by taking a longer time to develop and thus to emerge at a larger size (Timms, 1998). We found that the development on Kpodjiguegue took longer than on Californian blackeyed beans, but there was no difference in fecundity. This might indicate that Kpodjiguegue is indeed a less suitable host, but not unsuitable since the beetles were able to compensate for the drop in reproductive fitness.

In the two-choice experiment, the bean variety with the smooth testa was not preferred over the rougher Californian blackeyed bean. This is contradictory to the findings of Oigiangbe and Onigbinde (1996) who found that of nine cowpea varieties, the smooth skinned ones were preferred over rougher varieties. Besides, because the attachment of the egg to the bean surface would be facilitated, flat-cheeked seeds were preferred over rounder ones to oviposit on. Most eggs deposited by *C. maculatus* are laid on the cheek of the seed and most larvae develop in tunnels along the cheek (Ofuya, 1987). However, we found that the size of the beans did not influence the choice behaviour. Moreover, the Kpodjiguegue beans usually have a flatter cheek than the better-filled Californian blackeyed beans. For the Californian blackeyed variety, it was even observed that the smoothest seeds, that are usually small, were not preferred over rougher ones.

The colour of the seed coat did not influence oviposition in a no-choice situation (Nwanze and Horber, 1975). However, Chavan *et al.* (1997) found that when a choice was given to the closely related beetle species *Callosobruchus chinensis*, darker coloured seeds were preferred for oviposition over white seeds. In contrast, our results show a preference for seeds with a light colour especially in a choice situation.

Differences in the length of the developmental period can be caused by differences in age of the female parent, with eggs that are laid later in her life being less viable and taking a longer period for their development than earlier eggs (Nwanze and Horber, 1975). However, even in the two-choice test, eggs on Kpodjiguegue beans were laid by females of the same age group as eggs on Californian blackeyed beans. Therefore, the bean variety itself must be the cause of the longer developmental period.

The mass rearing in our laboratory is done on Californian blackeyed beans, but the Beninese strains were collected from Kpodjiguegue beans. The Southern Beninese strain had only been in the laboratory for a few generations before preliminary tests were done which had results comparable to the ones obtained here. If there had been any effects of the rearing procedures or an adaptive preference for the Californian blackeyed beans, this would have evolved within a maximum of two generations for the Southern Beninese strain.

The beetles of all strains preferred the Californian blackeyed beans if they were given the choice. Even in a no-choice situation, the beetles do slightly better on Californian blackeyed beans. These are a widely grown well-known variety. An additional advantage would be that Californian blackeyed beans are readily available from reliable suppliers with a known history of treatment. Since this variety is the more susceptible of the two to *Callosobruchus maculatus* damage, it would be the best variety to use for the evaluation of the effect of insecticides.

Acknowledgements

The research was sponsored by the Centre Béninois pour le Développement Durable. Beninese project partners of the Faculté de Science Agronomique of the National University of Benin are acknowledged for supplying Kpodjiguegue beans and for collecting the beetle strains. The critical comments of A. van Huis and M. Dicke are highly appreciated.

Chapter 3

Toxicity and repellence of African plants traditionally used for the protection of stored cowpea against *Callosobruchus maculatus*

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Abstract

In search for botanical products to control the main insect pest of stored cowpea, *Callosobruchus maculatus*, 33 traditionally used African plants were tested in the laboratory for their toxic and repellent effects against this beetle. Toxicity was evaluated measuring life history parameters in a no-choice situation. Powders of *Nicotiana tabacum*, *Tephrosia vogelii* and *Securidaca longepedunculata* significantly reduced the number of progeny. Repellence was evaluated observing the behaviour of female beetles exposed to treated and untreated beans in a linear olfactometer. *Clausena anisata*, *Dracaena arborea*, *T. vogelii*, *Momordica charantia* and *Blumea aurita* were repellent to the beetle, whereas *Chamaecrista nigricans*, *Azadirachta indica* and *Hyptis suaveolens* were attractive. Our results indicate that botanical products may provide effective control of *C. maculatus* in cowpea.

Key words: stored product pests, botanical insecticides, *Vigna unguiculata*, Coleoptera, life-history

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important crop for many subsistence farmers in tropical areas, especially in Africa. The green plant parts can be used as a vegetable or as fodder for cattle, whereas the seeds, the cowpea beans contain a high level of proteins and are used as human food (Phillips & McWatters, 1991).

In the field, the crop is susceptible to many pests (Singh *et al.*, 1990a). The dry, ripe seeds however, in the field or in storage are vulnerable to only few pests of which the cowpea weevil, *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae) is the most important insect pest. Infestation by this beetle commences in the field (Prevett, 1961), but most damage is done during storage. Over 90 % of the insect damage to cowpea seeds is caused by *C. maculatus* (Caswell, 1981). Infestation may reach 100% within 3 to 5 months of storage (Singh, 1977). The germination of the beans is negatively affected due to the

beetles' emergence holes (Baier & Webster, 1992). Damaged seeds loose weight and market value and they are generally unacceptable for human consumption (Javaid & Poswal, 1995).
To protect the stored beans against *C. maculatus*, many methods can be used. Traditionally the beans, if treated at all, are mixed or covered with materials that are available on the spot. They can be mixed with ash, sand, or other dry fine substances that can fill-up the space between the beans providing a barrier to insect movement (Golob & Webley, 1980). Fresh, dry or processed plant materials can be applied as insecticides or to repel the pest insects. These methods, in combination with a natural come and go of parasitoids, should keep the beetle infestation as low as possible. Nowadays, methods such as storage in airtight plastic or steel containers, application of chemical insecticides, gamma irradiation, freezing the beans or heating them, are some of the additional possibilities. However, most of these methods require high inputs, often unavailable and unaffordable for subsistence farmers. As for ash and sand, the main disadvantage is that to be effective they have to be applied in such large quantities that they are practical only for small amounts of beans (Wegmann, 1983). With the introduction of - often subsidised - chemical pesticides much of the traditional knowledge of the use of plant materials as pesticides is perishing (Koné, 1993). Moreover, the development of synthetic pesticides goes so fast that the spread of botanical insecticides is interrupted (Delobel & Malonga, 1987). Meanwhile *C. maculatus* continues to destroy stocks of beans that could feed hungry humans.
It is difficult to design chemicals which act specifically towards a given group of target insects (Wells *et al.*, 1993). Besides hazardous effects on natural enemies, the limited availability, dangers and costs associated with the use of synthetic insecticides and the problems of resistance of the insect against these products make it necessary to reconsider the potential of traditional methods which have stood the test of time (Golob & Webley, 1980). Another important advantage of traditional methods such as the application of plant materials over synthetic pesticides is that many of them are freely available at places where cowpea grows. Plant products could offer a solution for the damage done by *C. maculatus* and be non-damaging to non-target organisms including mammals and the beetle's natural enemies. The products are biodegradable and thus unlikely to have long-term hazardous effects on the environment.
Plant powders can have a protective effect on the beans based on several mechanisms. Plant material may produce odours that repel or confuse the adult beetle, which could prevent invasion or cause emigration from the treated stock if the possibility is given. When adult beetles leave the storage room before they can cause serious harm, insect damage will be minimised. For other plants, certain secondary metabolites are toxic to adult insects or to their eggs. Larvae and pupae of bruchid beetles are less sensitive to most crop protection methods, because they are protected by the bean in which they develop. Combinations of repellent and toxic effects are possible as well.

Many plants have been tested in laboratories for their toxic effects on storage beetles and few of them were tested for their repellent effect. Comparison of results obtained under laboratory conditions to the situation under actual storage conditions is problematic, but a hierarchy for the potential efficacy of plants can be established. Unfortunately, the outcomes of such tests are often contradictory to others and few authors have been able to recommend a certain plant or an application method (reviewed in Part I of this thesis).
A survey has been carried out in Benin (West Africa) to establish which plants are/were most often used in traditional storage practice for the protection of stored cowpea. For each of the six provinces of Benin about five of the most frequently used plant species were selected. These plants were tested in the laboratory for both their toxic and repellent effect against *C. maculatus*. If these plants would prove to be effective, their adoption as measures for crop protection by farmers would be easier as they were already used traditionally.

Material and methods

Beans

We used cowpea (*Vigna unguiculata*) of the variety California Blackeye, a variety susceptible to *C. maculatus* (Baker *et al.*, 1989). The beans were stored in a freezer at -18°C for a week and subsequently dried in a stove at 60°C for about a week to guarantee the absence of viable insects without having to use chemicals. The beans were stored in airtight plastic containers at room temperature before use. Only visually uninfested beans were used for the experiments.

Insects

Callosobruchus maculatus was collected in the north of Benin on local varieties of cowpea. The beetles were reared on cowpea (var. California Blackeye) in our laboratory for about a year (±14 generations) prior to the experiments. The rearing was done in a climate chamber at 30 ± 1 °C with a twelve-hour photoperiod at ambient relative humidity (50-80%). For the tests, newly emerged (1-1.5h) insects were used. For the repellence tests, female beetles were used that had been kept for an hour with a surplus of newly emerged males and were supposed to have mated.

Plant materials

Plants were collected and dried in Benin (West Africa) and Tanzania (East Africa) (see Table 1). The climatic conditions in the provinces of Benin are as follows: Atacora, in the Northwest and Borgou in the Northeast are in a Sahelien zone with one long rainy season. Atlantique and Ouémé in the South together form the humid coastal area with two rainy seasons, whereas Mono and Zou represent the centre of the country with a transition between the former two climates. For some plant species that were among the most

mentioned plants in more than one region, several samples were collected from different origins. After transport to the Netherlands, the plant samples were stored in plastic bags in the dark at 4 °C. Shortly before use, after warming up until room temperature, the dry plant material was powdered in a mortar. Powders were sieved through a 0.75-mm mesh before application to the beans.

TESTS

All tests were done in a climate chamber at 30 ± 2 °C with a 12-hour photoperiod at ambient relative humidity (50 – 80 %). Untreated beans were used as controls for every experiment.

Toxicity

For toxicity tests, 40.0 g beans and 1.00 g of plant powder (i.e. 25 g/kg) were thoroughly shaken in a 9-cm petri dish for two minutes. On these beans, two males and one female beetle were released. For every plant species, we used five replicates.

After 24 hours, the number of eggs was counted. The adult beetles were observed daily, and after their death, their life span in days was noted and the total amount of eggs was counted. The petri dishes were incubated under standard conditions to allow the eggs to develop into adults. Emerging F1 adults were counted, sexed and removed from the beans daily. Thus, information was obtained on the lifetime fecundity of the females and on the survival of the immature life stages of the beetle. Batches of two to five treatment sets (10-25 petri dishes) were tested simultaneously with a set of five untreated dishes as a control. In total, 75 controls were investigated in 15 batches. The data for each control set were compared to the mean of all controls in an ANOVA test. If no differences were found for the control set, the data from treatment sets tested simultaneously in this batch were compared to the data of other batches. If the control was statistically different, the data for the plants tested simultaneously with this control were compared to this deviating control only and analysed separately. To be able to always compare two samples of the same plant from different regions, these were always tested simultaneously. Toxicity tests were repeated only three times for the sample of *Securidaca longepedunculata* Borgou due to a lack of plant material. Knowing that most of the beetle eggs are laid in the first halve of the female beetles' life and that the sex ratio of the emerging beetles is never different from 50:50 (Boeke, unpublished results), the intrinsic rate of increase of the population per day, λ, was calculated according to Howes' (1953) method as $\lambda = np \exp(1/d+0.5*l)$. With n = the number of female eggs laid (half the total number of eggs), p = the proportion of eggs that mature, d = the development period in days and l = the oviposition period = (half the longevity of the female parent beetle).

Table 1: Names and origins of the plant materials tested against *Callosobruchus maculatus*.

Scientific name	Family	Local name	Plant part	Origin
Annona muricata L.	Annonaceae	Soursop	Leaves	Mono
Annona senegalensis Pers.	Annonaceae	Batoko / wild custardapple	Leaves	- Atacora - Borgou
Azadirachta indica Juss	Meliaceae	Neem	Leaves	- Atlantique - Zou - Tanzania
Blumea aurita (L.F) DC	Asteraceae	Faux tabac	Leaves	Zou
Capsicum frutescens L.	Solanaceae	Pepper	Fruits	Zou
Carica papaya L.	Caricaceae	Papaya	Leaves	Zou
Chamaecrista nigricans # (Vahl) Greene	Leguminosae-Caesalpinioideae	Moutounditimou	Leaves	Atacora
Clausena anisata (Willd.) Hook ex f. Benth.	Rutaceae		Leaves	Ouémé
Combretum micranthum G. Don	Combretaceae		Leaves	Atlantique
Crateva religiosa Forster f.	Capparaceae	Boumbari / sacred garlic pear	Leaves	Atacora
Cymbopogon citratus (DC. ex Nees) Stapf	Poaceae	Lemongrass	Leaves	Mono
Dracaena arborea (Wild.) Link	Liliaceae	Dragontree	Leaves	- Atlantique - Mono
Ficus exasperata Vahl	Moraceae		Leaves	Atlantique
Heliotropium indicum L.	Boraginaceae	Indian heliotrope	Twigs & flowers	Ouémé
Hyptis spicigera Lam.	Lamiaceae	Tinan menati / marubio	Leaves & flowers	- Atacora - Borgou
Hyptis suaveolens (L.) Poit.	Lamiaceae	Pignut	Leaves & flowers	Ouémé
Iboza multiflora (Benth) E. A. Bruse	Lamiaceae	Omushunshu	Leaves	Tanzania
Khaya senegalensis (Desr.) A. Juss	Meliaceae	African mahogany	Bark	Borgou
Momordica charantia L.	Cucurbitaceae	Bittergourd	Leaves	Mono
Moringa oleifera Lam.	Moringaceae	Horse radish tree	Leaves	Ouémé
Nicotiana tabacum L.	Solanaceae	Tobacco	Leaves	Tanzania
Ocimum basilicum L.	Lamiaceae	Sweet basil	Twigs & flowers	Ouémé
Opilia celtidifolia (Guil & Perr.) Endl.	Opiliaceae		Flowers	Borgou
Pergularia daemia (Forsskal) Chiov.	Asclepiadaceae	Pergularia	Leaves	Mono
Securidaca longepedunculata Fresen	Polygalaceae	Violet tree	Leaves	- Atacora - Borgou
Tagetes minuta L.	Asteraceae	Mexican marigold	Leaves	Tanzania
Tephrosia vogelii Hook f.	Leguminosae-Papilionoideae	Vogel's tephrosia	Leaves	Tanzania

Synonym = *Cassia nigricans*

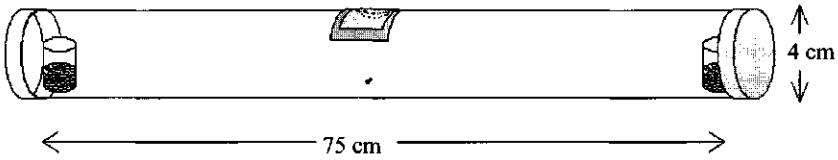


Figure 1: Olfactometer set-up. An individual female beetle was introduced in the centre of the tube. Her position was recorded after different periods since introduction. Treated and control beans were positioned at either end of the tube. For more information, see text.

Repellence

The repellent action of the plants was tested in an olfactometer (Figure 1), consisting of a 75-cm glass tube of 4 cm in diameter, with a 29-mm hole in the middle. At either end of the tube, a small jar was placed containing either 2.0 g untreated beans or 2.0 g of beans mixed with 0.010 g (i.e. 5 g/kg) plant powder. The hole in the middle was covered with gauze, whereas the ends of the tube were closed by putting a plastic petri dish against them. Air was gently (ca 1 m/s) sucked away from the centre of the tube to prevent the accumulation of plant odour in the tube.

One freshly mated female beetle was released in the middle of the tube through the hole. The beetles' behaviour was continuously observed for the first hour and its position was noted at least once an hour until 6 hours after the release. After 22 hours, the female was removed and the eggs on the beans in the jar she had entered were counted. Since the beetles did not move immediately and were not especially mobile during most of the experiment, they could be observed simultaneously in separate tubes. All repellence tests were replicated 36-46 times during two or three days with a new beetle for every repetition. Plants were tested in series, and in random order within one series. All plants were tested once before the second series. Treatment and control sides were randomly assigned.

The beetles that made a choice were divided in three groups: a) the ones that entered one of the jars containing the beans and thus had the possibility to lay eggs, b) the ones reaching an end (the last five centimetres) of the tube and c) the ones having chosen a side of the tube without reaching the end of that side of the tube. With time passing, more of the beetles made a choice.

Statistics

Data for the toxicity experiment were analysed using a one-way ANOVA and a subsequent Bonferroni post-hoc test. Data of the repellence experiment were analysed for their binomial distribution assuming a 50:50 distribution as the null-hypothesis. To check if there was any

effect of the day on which the experiments were done, for each treatment an R * C test of independence (Sokal & Rohlf, 1995) was done on the data of the separate days. For the numbers of eggs on beans in jars where a beetle had entered during the repellence experiments, a Mann-Whitney U test was performed.

Results

Toxicity

The results of the toxicity tests are shown in Table 2. None of the control sets showed differences with the mean of all controls concerning the number of eggs laid after 24 hours, the longevity of the parent beetles, the total number of eggs laid or the total number of beetles emerged. Beetles on beans treated with Capsicum frutescens, Tagetes minuta or Tephrosia vogelii laid fewer eggs in the first 24 hour period than beetles on untreated beans. The longevity of the parent beetles and the total number of eggs were reduced only by T. vogelii. Compared to the controls, a smaller number of beetles emerged from beans treated with, Securidaca longepedunculata Borgou or T. vogelii than from control beans. From the beans treated with Nicotiana tabacum, not a single beetle emerged.

The percentage of eggs that did not develop into adults was higher for the controls of batch A than for the controls of other batches. *S. longepedunculata* Borgou, *T. vogelii* and *N. tabacum* caused higher percentages mortality of the developing stages of the beetle. For the separately analysed batch (batch A), the percentage mortality of immature stages on beans treated with *Hyptis suaveolens*, *Clausena anisata* and *Moringa oleifera* was lower than in the control set.

The intrinsic rate of increase of the insect population was equal for all control sets. The value was significantly lower than on control beans for beetles developing on beans treated with *Annona senegalensis* Atacora, *N. tabacum*, and *T. vogelii*.

Repellence

Once the beetles had entered a jar with beans at the end of either side of the tube, they did not leave it before the end of the experiment, 22 hours after release. A day-effect ($P < 0.05$) was found for *Annona muricata, Annona senegalensis* Borgou, *Combretum micranthum, Ficus exasperata, Blumea aurita* and *Securidaca longepedunculata* Atacora. Since these day-effects were never contradictory (repellent one day, attractive the other), and were caused only by a change in the extent of the effect, they were not taken into account in further analyses.

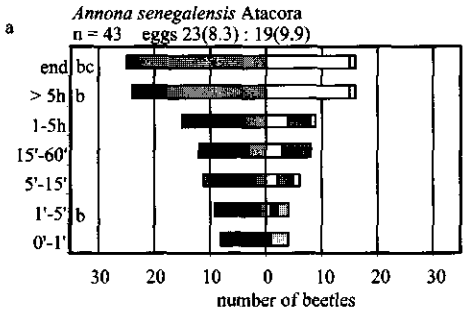
More eggs were laid on untreated control beans than on beans treated with *Capsicum frutescens, Clausena anisata, Moringa oleifera* ($P < 0.05$) and *Tephrosia vogelii* ($P < 0.01$). For *Securidaca longepedunculata* Atacora, more eggs were laid on the treated beans than on the control beans ($P < 0.05$).

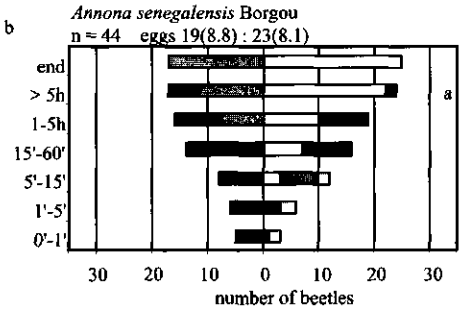
Table 2: Results, (means of n measurements ± stdev) of toxicity tests of cowpea beans mixed with plant powder against *Callosobruchus maculatus*. Numbers in columns followed by the same letter are not significantly different.

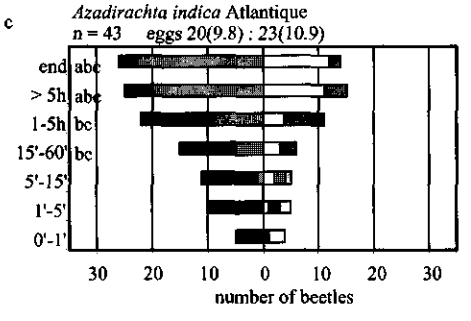
Treatment	Batch	N	Number of eggs	
			Day 1	Total
Control	A-M	75	22.8 ± 7.49 abde	75.8 ± 20.85 a
Annona muricata	H	5	21.2 ± 4.97 abcde	69.2 ± 14.94 a
Annona senegalensis Atacora	B	5	17.4 ± 12.46 cde	54.0 ± 30.15 a
Annona senegalensis Borgou	B	5	24.4 ± 4.62 abcde	71.2 ± 16.83 a
Azadirachta indica Atacora	G	5	18.2 ± 3.19 abcde	79.4 ± 14.57 a
Azadirachta indica Tanzania	G	10	20.8 ± 5.77 abcde	76.6 ± 22.74 a
Azadirachta indica Zou	G	5	12.8 ± 3.03 cde	71.2 ± 11.50 a
Blumea aurita	J	5	19.2 ± 3.42 abcde	80.0 ± 9.54 a
Capsicum frutescens	M	5	11.0 ± 6.25 cd	77.8 ± 18.91 a
Carica papaya	H	5	22.2 ± 4.38 abcde	71.0 ± 14.30 a
Chamaecrista nigricans	D	5	26.6 ± 3.21 abde	85.2 ± 13.54 a
Clausena anisata	A	5	15.0 ± 9.82 cde	61.0 ± 28.36 a
Combretum micranthum	I	5	11.6 ± 3.05cd	64.0 ± 13.15 a
Crateva religiosa	D	5	28.2 ± 9.65 abe	78.4 ± 24.43 a
Cymbopogon citratus	M	5	18.4 ± 2.51 abcde	94.0 ± 15.75 a
Dracaena arborea Atlantique	L	5	14.2 ± 3.35 cde	85.4 ± 17.99 a
Dracaena arborea Mono	L	5	19.0 ± 6.71 abcde	84.2 ± 9.78 a
Ficus exasperata	J	5	33.8 ± 13.50 abe	81.6 ± 24.39 a
Heliotropium indicum	A	5	26.4 ± 1.14 abde	60.6 ± 10.97 a
Hyptis spicigera Atacora	C	5	14.0 ± 5.15 cde	73.0 ± 10.68 a
Hyptis spicigera Borgou	C	5	24.8 ± 2.68 abde	84.6 ± 11.19 a
Hyptis suaveolens	A	5	20.6 ± 3.05 abcde	71.0 ± 10.30 a
Iboza multiflora	K	5	15.2 ± 5.36 cde	87.2 ± 12.93 a
Khaya senegalensis	F	5	21.6 ± 4.16 abcde	88.0 ± 24.94 a
Momordica charantia	J	5	23.2 ± 5.54 abcde	89.2 ± 19.41 a
Moringa oleifera	A	5	26.0 ± 3.74 abde	56.4 ± 15.77 a
Nicotiana tabacum	K	5	16.6 ± 4.67 cde	73.2 ± 22.33 a
Ocimum basilicum	A	5	17.6 ± 5.32 bcde	54.6 ± 11.78 a
Opilia celtidifolia	F	5	19.4 ± 4.72 abcde	80.0 ± 24.63 a
Pergularia daemia	I	5	12.8 ± 3.63 cde	64.8 ± 11.12 a
Securidaca Atacora	E	5	25.7 ± 3.06 abcde	84.0 ± 11.53 a
Securidaca Borgou	E	3	22.0 ± 7.25 abcde	91.8 ± 10.31 a
Tagetes minuta	M	5	8.4 ± 3.58 cd	87.8 ± 20.07 a
Tephrosia vogelii	K	5	1.8 ± 1.48 cd	2.4 ± 1.67 b

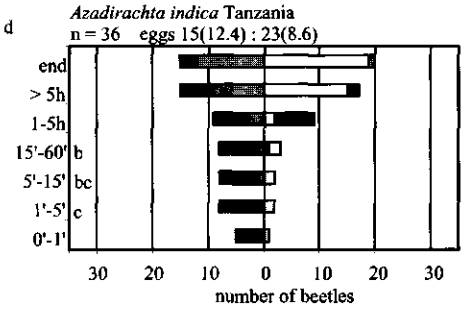
Longevity (days)		Total emerged	Percentage mortality	λ value * 100
Female	Males			
4.9 ± 0.84 ab	5.1 ± 0.97 ab	61.6 ± 18.65 a	18.9 ± 12.98 ab	114.0 ± 2.5 a
4.2 ± 0.45 abc	5.1 ± 0.57 ab	48.0 ± 8.34 ab	29.9 ± 6.21 a	113.0 ± 1.0 ab
4.6 ± 0.55 abc	4.9 ± 0.99 ab	44.8 ± 25.85 ab	32.9 ± 39.18 a	9.18 ± 51.3 b
4.4 ± 0.55 abc	4.9 ± 0.99 ab	52.6 ± 5.77 ab	24.4 ± 10.31 a	114.5 ± 0.9 ab
5.0 ± 1.00 abc	5.2 ± 0.63 ab	62.0 ± 13.58 ab	21.9 ± 7.61 a	114.3 ± 0.9 ab
4.9 ± 0.99 ab	5.1 ± 0.51 ab	60.8 ± 23.39 a	21.4 ± 14.75 a	114.1 ± 1.2 ab
4.6 ± 0.55 abc	5.1 ± 0.32 ab	62.0 ± 14.16 ab	13.4 ± 10.90 a	113.7 ± 0.9 ab
5.4 ± 1.34 ab	4.0 ± 0.94 a	63.6 ± 10.21 a	20.6 ± 7.57 a	113.8 ± 0.6 ab
5.2 ± 1.30 ab	5.6 ± 0.70 ab	66.0 ± 9.77 a	13.4 ± 9.95 a	113.8 ± 0.6 ab
3.8 ± 0.45 ac	4.2 ± 0.42 a	62.8 ± 8.87 ab	10.3 ± 11.17 a	113.7 ± 1.1 ab
5.4 ± 1.67 ab	4.7 ± 1.34 ab	65.6 ± 12.60 a	22.5 ± 12.35 a	115.1 ± 0.5 ab
5.0 ± 1.22 abc	5.3 ± 0.67 ab	49.4 ± 23.46 ab	19.8 ± 4.25 c	112.9 ± 3.2 ab
3.8 ± 0.45 ac	4.8 ± 1.23 ab	51.8 ± 8.41 ab	17.1 ± 16.40 a	113.5 ± 1.2 ab
4.2 ± 0.45 abc	4.6 ± 1.08 ab	68.6 ± 22.80 a	13.0 ± 6.54 a	115.4 ± 2.1 ab
6.4 ± 1.34 ab	6.1 ± 1.20 ab	80.0 ± 10.65 a	14.4 ± 6.06 a	114.2 ± 0.6 ab
5.6 ± 1.82 ab	5.0 ± 0.82 ab	74.0 ± 16.02 a	13.4 ± 3.74 a	114.0 ± 0.7 ab
5.0 ± 0.71 abc	4.7 ± 1.16 ab	69.4 ± 14.78 a	18.2 ± 9.49 a	113.9 ± 1.8 ab
4.4 ± 0.89 abc	4.6 ± 0.84 ab	67.4 ± 20.23 a	17.0 ± 4.11 a	114.0 ± 1.6 ab
4.4 ± 0.55 abc	5.0 ± 0.47 ab	43.4 ± 8.71 ab	28.2 ± 9.50 bc	113.3 ± 1.4 ab
5.0 ± 0.71 abc	5.5 ± 0.85 ab	60.6 ± 11.01 ab	17.1 ± 7.00 a	114.8 ± 0.9 ab
5.2 ± 0.45 ab	5.4 ± 0.97 ab	61.2 ± 9.68 ab	27.3 ± 8.92 a	114.9 ± 1.1 ab
5.3 ± 1.50 ab	5.3 ± 1.04 ab	45.4 ± 26.63 ab	20.1 ± 5.81 c	114.9 ± 0.1 ab
4.6 ± 0.89 abc	5.1 ± 0.74 ab	71.0 ± 13.34 a	18.6 ± 10.01 a	114.1 ± 1.3 ab
5.4 ± 1.14 ab	4.5 ± 0.85 ab	70.2 ± 21.41 ab	20.5 ± 9.23 a	115.4 ± 1.4 ab
5.2 ± 1.10 ab	5.4 ± 1.08 ab	78.2 ± 17.61 a	12.2 ± 8.46 a	115.0 ± 0.3 ab
4.0 ± 0.71 abc	5.0 ± 0.47 ab	43.2 ± 7.76 ab	21.3 ± 11.96 c	114.0 ± 0.9 ab
6.0 ± 1.41 ab	5.2 ± 1.03 ab	0 ± 0 bc	100 ± 0 d	0 ± 0 c
4.4 ± 0.55 abc	5.4 ± 0.52 ab	41.4 ± 17.67 abc	26.9 ± 17.55 bc	112.7 ± 1.8 ab
5.2 ± 2.39 ab	4.9 ± 0.99 ab	70.6 ± 23.58 a	12.7 ± 5.89 a	115.5 ± 1.9 ab
4.2 ± 0.84 abc	4.6 ± 0.84 ab	52.8 ± 10.55 ab	18.8 ± 3.11 a	113.8 ± 1.4 ab
5.7 ± 1.15 ab	4.8 ± 0.98 ab	68.3 ± 13.20 ab	18.9 ± 8.46 a	114.8 ± 0.3 ab
5.6 ± 0.89 ab	5.0 ± 0.47 ab	21.8 ± 16.02 bc	77.2 ± 15.33 d	108.2 ± 5.3 ab
5.4 ± 1.14 ab	5.5 ± 0.85 ab	72.6 ± 9.21 a	15.6 ± 10.92 a	113.9 ± 0.4 ab
2.6 ± 2.07 b	1.0 ± 0.00 c	0.6 ± 0.89 bc	75.0 ± 28.87 d	40.0 ± 54.8 d

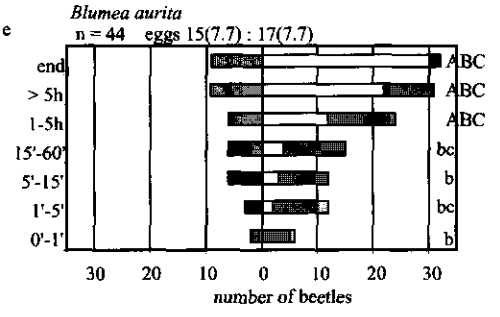
No significant preference for any side of the tube at any time during the experiment was found for *Annona muricata*, *Azadirachta indica* Zou, *Cymbopogon citratus*, *Hyptis spicigera* Borgou, *Khaya senegalensis*, *Moringa oleifera*, *Ocimum basilicum*, *Pergularia daemia*, or *Tagetes minuta*. For the other treatments, we compare the treatment at one side of the tube to the untreated control at the other side. The results of these tests are shown in Figures 2 a – x.
Eleven plants had an attractive effect, at least at one moment during the experiment. For *Annona senegalensis* Atacora, *Azadirachta indica* Tanzania, *Crateva religiosa*, *Ficus exasperata* and *Hyptis spicigera* Atacora more beetles chose the treatment side and reached the treatment end of the tube at some point during the experiment. No effect was found at the end of the experiment except for *A. senegalensis* Atacora. The number of beetles that entered the jars with beans was never different for the control and the treatment side for any of these treatments.
The number of beetles in treatment jars was higher at some point during the experiment for *Capsicum frutescens* and for *Securidaca longepedunculata* Atacora and Borgou. Only for *S. longepedunculata* Atacora was this difference still found at the end of the experiment.
For *Chamaecrista nigricans*, the attractive effect with more beetles at the treatment side, at the treatment end of the tube and in the treatment jars, was significant at the end of the experiment only. For *Azadirachta indica* Atlantique it became apparent within an hour and for *Hyptis suaveolens*, the effect was significant after five minutes until the end of the experiment.
The other thirteen plants had a repellent effect at least at one moment during the experiment. More beetles were present at the control side and end after five minutes for *Dracaena arborea* Atlantique and at the end of the experiment for *Iboza multiflora* and *Nicotiana tabacum*. For *Annona senegalensis* Borgou and *Opilia celtidifolia*, there were more beetles in the control jars at some point during the experiment, but at the end of the experiment, this pattern had disappeared. *Heliotropium indicum* led to repellence with more beetles choosing the control side of the tube and entering the control jars, but again no effect was left at the end of the experiment.
For the other treatments, the differences were significant until the end of the experiment. For *Carica papaya*, more beetles chose the control side and end of the tube. More beetles were present in the control jars or at the control side or end of the tube for *Clausena anisata*, *Combretum micranthum*, *Dracaena arborea* Mono, *Momordica charantia* and *Tephrosia vogelii*. From the first minute on until the end of the experiment, more beetles were at the control end for the *Blumea aurita* treatment.

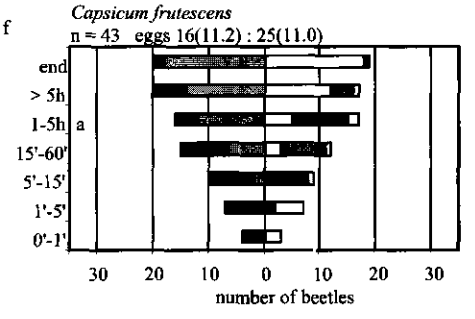

a
Annona senegalensis Atacora
n = 43 eggs 23(8.3) : 19(9.9)
end bc
> 5h b
1-5h
15'-60'
5'-15'
1'-5' b
0'-1'
30 20 10 0 10 20 30
number of beetles

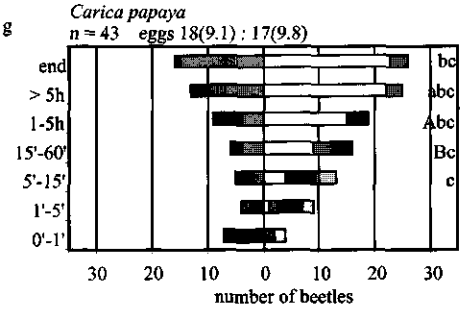

b
Annona senegalensis Borgou
n = 44 eggs 19(8.8) : 23(8.1)
end
> 5h
1-5h
15'-60'
5'-15'
1'-5'
0'-1'
a
30 20 10 0 10 20 30
number of beetles

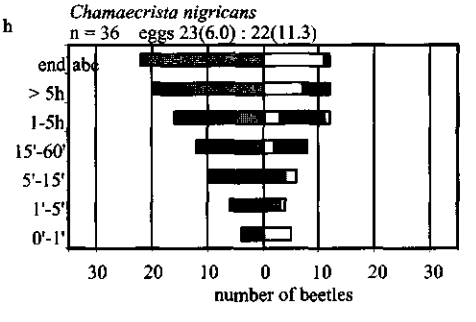

c
Azadirachta indica Atlantique
n = 43 eggs 20(9.8) : 23(10.9)
end abc
> 5h abc
1-5h bc
15'-60' bc
5'-15'
1'-5'
0'-1'
30 20 10 0 10 20 30
number of beetles


d
Azadirachta indica Tanzania
n = 36 eggs 15(12.4) : 23(8.6)
end
> 5h
1-5h
15'-60' b
5'-15' bc
1'-5' c
0'-1'
30 20 10 0 10 20 30
number of beetles

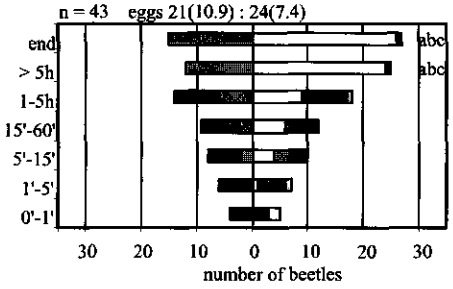

e
Blumea aurita
n = 44 eggs 15(7.7) : 17(7.7)
end
> 5h
1-5h
15'-60'
5'-15'
1'-5'
0'-1'
ABC
ABC
ABC
bc
b
bc
b
30 20 10 0 10 20 30
number of beetles


f
Capsicum frutescens
n = 43 eggs 16(11.2) : 25(11.0)
end
> 5h
1-5h a
15'-60'
5'-15'
1'-5'
0'-1'
30 20 10 0 10 20 30
number of beetles

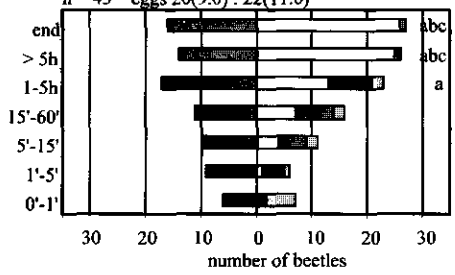

g
Carica papaya
n = 43 eggs 18(9.1) : 17(9.8)
end
> 5h
1-5h
15'-60'
5'-15'
1'-5'
0'-1'
bc
abc
Abc
Bc
c
30 20 10 0 10 20 30
number of beetles


h
Chamaecrista nigricans
n = 36 eggs 23(6.0) : 22(11.3)
end abc
> 5h
1-5h
15'-60'
5'-15'
1'-5'
0'-1'
30 20 10 0 10 20 30
number of beetles

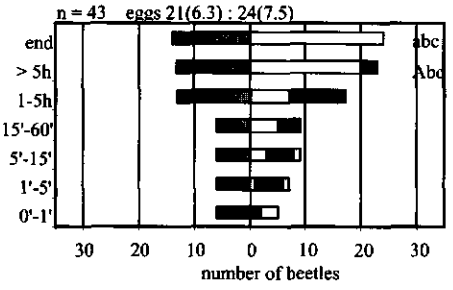
i *Clausena anisata*
n = 43 eggs 21(10.9) : 24(7.4)



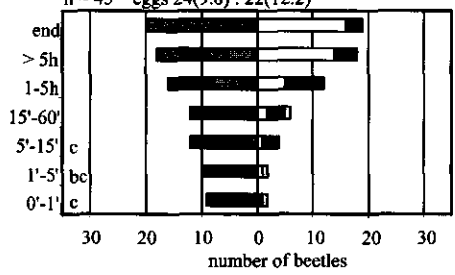
m *Dracaena arborea* Mono
n = 43 eggs 20(9.0) : 22(11.0)



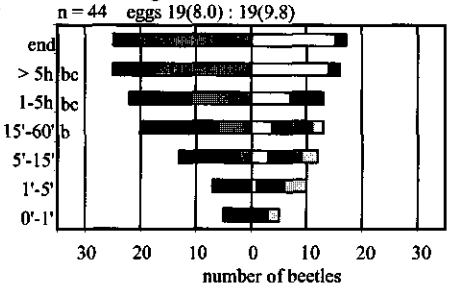
j *Combretum micranthum*
n = 43 eggs 21(6.3) : 24(7.5)



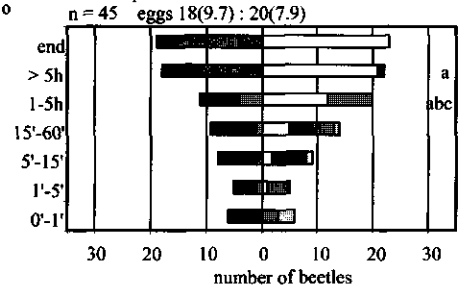
n *Ficus exasperata*
n = 43 eggs 24(9.8) : 22(12.2)



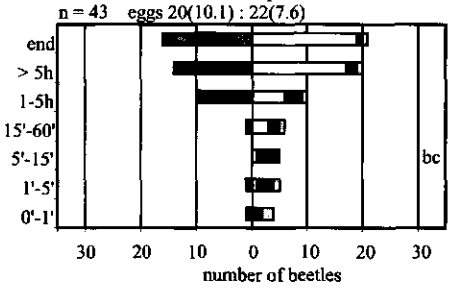
k *Crateva religiosa*
n = 44 eggs 19(8.0) : 19(9.8)



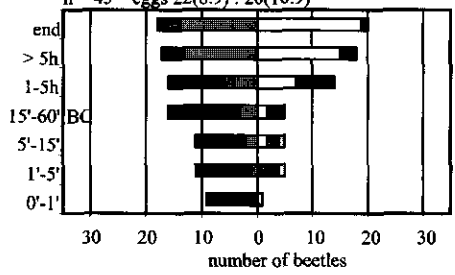
o *Heliotropium indicum*
n = 45 eggs 18(9.7) : 20(7.9)



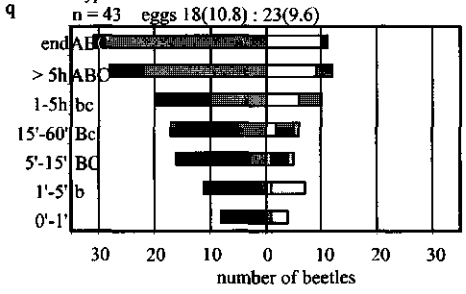
l *Dracaena arborea* Atlantique
n = 43 eggs 20(10.1) : 22(7.6)



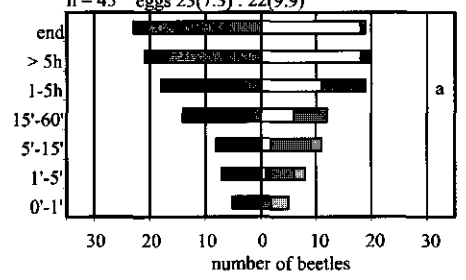
p *Hyptis spicigera* Atacora
n = 43 eggs 22(8.9) : 20(10.9)



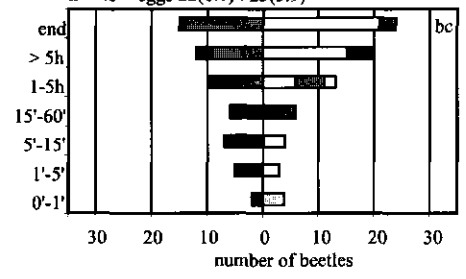
q *Hyptis suaveolens*
n = 43 eggs 18(10.8) : 23(9.6)



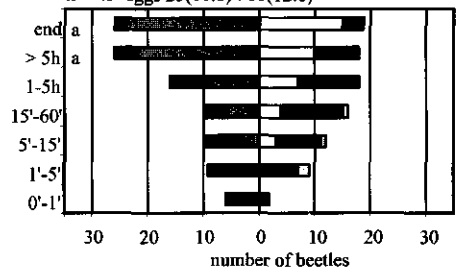
u *Opilia celtidifolia*
n = 45 eggs 23(7.3) : 22(9.9)



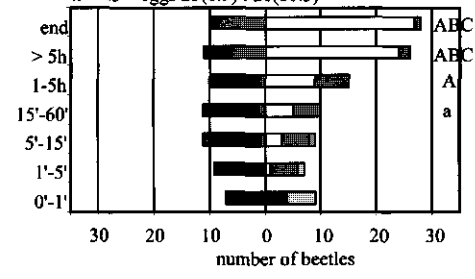
r *Iboza multiflora*
n = 43 eggs 22(6.1) : 23(5.9)



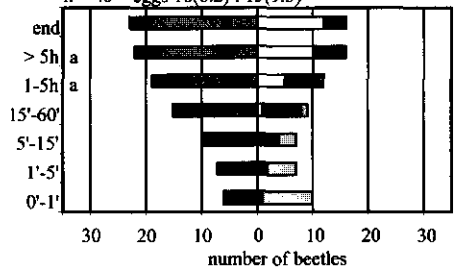
v *Securidaca longepedunculata* Atacora
n = 45 eggs 23(11.8) : 16(12.8)



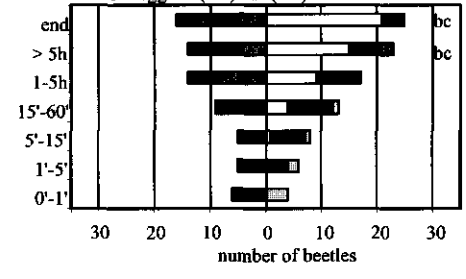
s *Momordica charantia*
n = 43 eggs 23(8.9) : 21(10.3)



w *Securidaca longepedunculata* Borgou
n = 43 eggs 16(8.2) : 15(9.5)



t *Nicotiana tabacum*
n = 43 eggs 17(8.8) : 21(7.6)



x *Tephrosia vogelii*
n = 36 eggs 3(2.7) : 17(9.9)

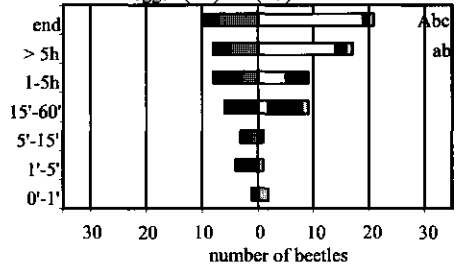


Figure 2 a-x: Results of olfactometer tests. At the left side the treatment is represented, the control side is shown at the right. The number of beetles is represented that was present ■ = in the jar with treated beans, ■ = at the treatment end of the olfactometer tube, ▩ = at the treatment side of the tube, □ = in the control jar, ■ = at the control end, □ = at the control side. Beetles that did not make a choice are not represented in the figure. a (& A) = more beetles in the jar with beans at this side of the olfactometer tube $P< 0.05$ (& $P< 0.01$), b (& B) = more beetles at this end of the tube $P < 0.05$ (& $P< 0.01$), c (& C) = more beetles at this side of the tube $P < 0.05$ (& $P < 0.01$). The number of eggs (Treatment (stdev): Control (stdev)) is the mean number of eggs in all jars where a beetle had entered.

Discussion

From our tests the most promising plant, with both toxic and repellent activity is *Tephrosia vogelii*. The repellent effect of *Nicotiana tabacum* is less profound, but here the complete ovicidal effect adds to its efficacy. *Blumea aurita* had a strong repellent effect, but it had no toxic effect at all. For *Securidaca longepedunculata* Borgou, we found a toxic effect whereas the powder had a tendency to attract the beetles.

The plants tested were selected in close co-operation with farmers. They are or were all traditionally used to protect stored cowpea against *Callosobruchus maculatus*. Strangely, in our laboratory experiments, we found positive effects on the beetle for some of the plants. For example, *Hyptis suaveolens* was very attractive and more of the eggs developed into adults than on untreated beans.

For some plant species, several samples were tested which resulted in some striking differences from sample to sample. *Dracaena arborea* from Mono was repellent to the beetles, whereas the same species harvested in Borgou was hardly effective. One of the samples of *Azadirachta indica* was clearly attractive to the beetles, whereas the others were less so or not at all. *Securidaca longepedunculata* Atacora did not have any toxic effects, whereas *S. longepedunculata* Borgou caused a high mortality in immature stages of the beetle. These differences in activity between samples of the same plant might have been caused by the growing conditions of the plants. Plants grown under different light qualities or intensities, with different availability of water and nutrients can contain different concentrations of secondary metabolites. Furthermore, ecotypes with altered insecticidal properties could have evolved under conditions where insect damage is more severe (Schoonhoven *et al.*, 1998). Differences in handling after collection of the plant material could have contributed to these findings as well.

For adult beetles, the determination of the time of death is possible if one knows the behaviour of the beetles. The beetles tend to exhibit a stupor response upon slight

disturbance and during the last days of their lives they can move only their legs and antennae. The day of death was here determined as the day the antennae and legs were not moved upon gentle disturbance with a forceps. Fresh beetle eggs are colourless and after a few days, the developing larva can be seen inside them. After hatching, the eggs turn milky white. Death of the larva inside the egg before hatching can thus be recognised by checking the colour of the egg. For the developmental stages inside the bean, several larval stages and the pupa, death cannot be determined accurately. In the California blackeye cowpea variety we used, which produces big yellowish seeds, the tunnel that is dug by the developing larva can be seen a few days before emergence of the adult beetle as a bluish spot under the seed coat. From the size and the clearness of this spot, the stage of development can be guessed, but more cannot be seen of the developing larva if the bean is to be kept intact. If no beetles emerged from the beans anymore for five days, the remaining larvae were considered dead.

In both the toxicity and the repellence test, the plants that proved to be effective against *C. maculatus*, are not the well-known toxic plants that appear often in the literature. *Azadirachta indica, Ocimum basilicum* and *Hyptis spicigera* are the most often tested plants of our list. The most effective ones such as *Tephrosia vogelii, Nicotiana tabacum, Securidaca longepedunculata, Blumea aurita* and *Momordica charantia* were tested much less often or never at all (Part I of this thesis). For *B. aurita, Moringa oleifera*, and *Dracaena arborea* no scientific information is available on their effects on bean beetles.

The ripe fruits of *Capsicum frutescens* have insecticidal, repellent, antifeedant and fumigant properties (Stoll, 1986). We found that on beans treated with *C. frutescens* fewer eggs were laid after 24 hours but that the plant had a slightly attractive effect on the beetles. Literature data on the effects of the fruits of *C. frutescens* are ambiguous. At 10 g/kg *C. frutescens* completely prevented oviposition by *C. maculatus* (Onu & Aliyu, 1995), whereas at the much higher dose of 2 g/20 beans it was found not to affect the development of the beetle (Dabire, 1985). Treatment of beans with *C. frutescens* at 20 g/kg had no effect on the number of eggs laid or the weight loss of beans (Javaid & Poswal, 1995). However, Morallo-Rejesus *et al.* (1990) found that *C. frutescens* at 0.03-0.12 %, caused complete mortality of adult bruchid beetles within five days, even at 6 months after application.

We found an attractive effect of *Chamaecrista nigricans* and no toxic effect. Others found that bruchid beetle oviposition (Dabire, 1985; Van Huis, 1991) and egg hatch are hampered by *C. nigricans* (De Groot, 1997; Stoll, 1986) and treated beans are much less prone to damage by newly hatched weevil larvae (De Groot, 1991).

Leaves of *Clausena anisata* contain large amounts of methyl chavicol, limonene and (*E*)-anethole (Ayedoun *et al.*, 1997). The plant is used for medical purposes against rheumatism, malaria, and influenza and as a heart tonic, an anthelminthic, a parasiticide, or a purgative. It contains coumarins with an antifeedant effect on insects (Gebreyesus & Chapya, 1983).

Leaf powder of *Combretum apiculatum* had some insecticidal effect against *C. maculatus*. Fewer eggs were laid, and fewer adults emerged. The toxicity to mammals is still to be

evaluated (Javaid & Mpotokwane, 1997). For *C. micranthum*, no insecticide or repellent effects have been reported yet.

Treatment with *Hyptis suaveolens* is usually found to be effective against insects (Adedire & Lajide, 1999; Belko, 1994; Fatope *et al.*, 1995) or not invoking any effect (Ajayi *et al.*, 1987). Leaves of *Azadirachta indica* and *Chamaecrista nigricans* have been reported to be non-effective or impairing a negative influence against *C. maculatus* (Fatope *et al.*, 1995; Dabire, 1991; Echendu, 1991; Mahgoub, 1995). The attractant effects we found for these plants remain unexplained.

Treatment of beans with powders of *Momordica* spp., *Cymbopogon citratus* or *Ficus exasperata* did not have any effect on the number of eggs and the percentage of them that hatched (Ofuya, 1990). The leaves of *Momordica charantia* contain momordicines and their derivatives, which have a feeding deterrent activity even on beetles species specialised on Cucurbitaceous plants (Abe & Matsuda, 2000). The strong repellent effect we found here against the cowpea weevil was not reported earlier.

Nicotiana tabacum caused no reduction of the number of eggs laid, but the development of the eggs stopped before the larva could penetrate the bean. Other authors found different results. Ofuya (1990) found that the number of eggs laid by *C. maculatus* females on beans treated with *N. tabacum* powder and the percentage of the eggs that hatched were lower than on untreated control beans. Rahman (1990) reported that *N. tabacum* did not affect the development of bruchid beetles. The egg mortality on beans treated with this plant was lower than for any of the other treatments he used. The data presented by Rahman are peculiar because nicotine, an active component of *N. tabacum* is a strong organic poison which acts as a contact-, stomach- and respiratory poison with insecticidal, repellent, fungicidal and acaricidal effects (Stoll, 1986).

We found an increased mortality of immature stages for one of the samples of *Securidaca longepedunculata*. The plant contains saponins that caused high larval and nymphal mortality and a reduced fecundity of larvae that managed to develop into adult *Spodoptera frugiperda* (Hubrecht *et al.*, 1989). Application of powder of *S. longepedunculata* to beans reduces or inhibits emergence and damage by *C. maculatus* (Anonymous, 1994).

The active product of *Tephrosia vogelii*, tephrosine (oxydegueline) is present in all plant parts. It is an absorption poison with no damaging effect for warm-blooded animals (Nzambi & Nagahuedi, 1993). The plant is also a source of rotenone (Delobel & Malonga, 1987) which has a low toxicity to warm-blooded animals and is biodegradable (Kaposhi, 1992). It is generally effective as an insecticide. We found that the adult beetles died soon after they came into contact with the plant powder and of the few eggs they managed to lay, very few developed into adults.

The differences in effect between our results and the ones cited from other publications could be due to differences of the used doses. In our experiment there was a factor 5 difference in concentration of the plant products between the toxicity and the repellence test.

Compared with doses used by other authors the 5 g/kg as used in the repellence tests, is quite low a concentration. The concentration used in the toxicity tests (25 g/kg), is in the same order of magnitude as the concentrations used in other studies (Part I of this thesis).
We know very little about the residual activity as insecticides of the plant powders tested here. The tests were done immediately after preparation of the powder and monitoring lasted only one beetle generation. The plant material that was ground for the tests was stored for at least a few months before use. The effect of fresher plants might be stronger than for the products used here.
When traditional methods such as the use of plant materials as insecticides, are brought to the laboratory for evaluation, the results appear to vary enormously. Some of the plants we tested here did not have any effect on the beetle or were attractive or causing the beetles to produce more offspring than on untreated beans. However, if the right material is selected, the method of using materials available in the neighbourhood of the cowpea field as protective agents for the seeds seems promising. *Nicotiana tabacum* completely prevented the emergence of a next generation, whereas the combined repellent and toxic effects of *Tephrosia vogelii* prevented oviposition and affected the beetle's lifecycle. Promising plant species will be investigated further for their effect on the beetles' natural enemies. The effects of the treatments on the stored product, the duration of the effect and the effect on consumers of the beans should also be evaluated. Our study indicates that cheap, sustainable protection of cowpea against storage insects may be feasible using traditional botanical products.

Acknowledgements

The research was financed by the Centre Béninois pour le Développement Durable. We thank the farmers in Benin for their co-operation and the project partners in Benin for the interviews with the farmers and the collection and packing of the plant materials.

Chapter 4

Efficacy of plant extracts against the cowpea beetle, *Callosobruchus maculatus*

S.J. Boeke, C. Barnaud, J.J.A. van Loon, D.K. Kossou, A. van Huis, M. Dicke

Abstract

Traditionally, African farmers used plants to protect stored cowpea from insect attack. To evaluate the efficacy of this practice, commonly used plant species were tested in the laboratory for their effects against the cowpea beetle, *Callosobruchus maculatus*. The plants that proved effective as powders were extracted with water and the extracts were tested for their possible toxic and repellent effects. Thirteen volatile oils, two non-volatile oils and eight slurries, all extracted from plants, were tested for their toxic and repellent effects. Application of volatile oils led in most cases to a reduced number of eggs on treated beans. The volatile oils of *Cymbopogon nardus* and *C. schoenanthus* caused the majority of the eggs not to develop into adult beetles. Repellent effects were found for *Clausena anisata*, *Cymbopogon citratus*, *Cymbopogon nardus*, a mixture of *Cymbopogon citratus* and *Cymbopogon flexuosus*, *Hyptis spicigera*, *Tagetes minuta* and for two samples of *Ocimum basilicum*. Non-volatile oils were not repellent and had no effect on the number of eggs laid, but the development of these eggs was hampered, most so by *Azadirachta indica* oil. None of the slurries had a toxic effect on the beetles, but the slurries obtained from *Carica papaya*, *Dracaena arborea* and *Tephrosia vogelii* were repellent, whereas the slurry from *Azadirachta indica* leaves was attractive. Oils, both volatile and non-volatile were easily extracted from plant material and showed promising results as a protective agent for stored cowpea.

Key words: Toxicity, repellence, Coleoptera, stored product pests, botanical insecticides, extracts, *Vigna unguiculata*, olfactometer, life-history

Introduction

The bruchid beetle *Callosobruchus maculatus* Fabricius causes more than 90 % of the insect damage of stored seeds of cowpea, *Vigna unguiculata* (L.) Walp. (Caswell, 1981). This beetle can destroy a complete harvest of cowpea beans within 5 months of storage (Singh, 1977). To prevent this, chemical pesticides are commonly applied, but in the quest for a

safe, cheap, easily applicable protection method for stored cowpea against *C. maculatus*, plant materials have proven their efficacy as insecticides as well (Part I of this thesis). Such plant materials, mixed with the seeds as powders, extracts or oils, can act as repellents or as toxicants against the beetle.

From previous research, powders of several plants traditionally used in Benin, West Africa, appeared to be repellent to adult beetles or to have toxic effects on various developmental stages of *C. maculatus* (Chapter 3). The plants were effective to varying extents, but their effects could probably be enhanced if the active compounds were concentrated. Extracts have been shown to be more effective than the powders they were obtained from (Mahgoub, 1992; Mbata *et al.*, 1995). The same has been found for volatile oils (Adebayo and Gbolade, 1994; Olaifa and Erhun, 1988) and for non-volatile oils (Lale and Abdulrahman, 1999) as compared to the powders they were extracted from. Here, we investigate if, with a single, easily performed extraction we can enhance the effect of the traditionally used plants that, as powders, proved effective against *C. maculatus*. Aqueous extracts were made and tested for their efficacy as toxic and repellent agents against the cowpea beetle. In this way we aim to develop a plant-based protection method that would be available for low resource farmers in Benin.

Material and methods

Beetles

Callosobruchus maculatus was collected on cowpea seeds in Northern Benin in 1999. The beetles were reared in the laboratory on the cowpea variety Californian Blackeyed in a climate chamber at 30 ± 1 °C with ambient relative humidity (50-80%) and a 12-hour photoperiod. For the experiments, newly emerged (1-1.5 h) beetles were used. For the repellence tests males and females were kept together for an hour after their emergence. The females that were used in these experiments were supposed to have mated.

Distillation of plant material

Most of the plant samples mentioned in Table 1 were collected and dried while protected from sunlight, in Benin, West Africa or in Tanzania (*Nicotiana tabacum*, *Tagetes minuta* and *Tephrosia vogelii*). The dry material was transported to the Netherlands and stored for several months at 4 °C until use.

For the extraction process, a Clevenger distillation set-up was used. According to availability, 25-125 g of the dry plant material was put in a flask and enough distilled water was added to cover the material. The flask was heated so that vapours came up that condensed in the water cooler. The condensed vapour flowed into a reservoir that served to refill the flask. Volatile oil assembled as a layer floating on the water in the reservoir and could be collected after the distillation process. If no oil layer was formed, whitish clouds

Table 1: Names and origins of the plant materials tested against *Callosobruchus maculatus*.

Scientific name	Family	Local name	Material	Origin [1]
Azadirachta indica Juss.	Meliaceae	Neem	Seed oil	Benin
			Slurry	Benin Zou distilled
Blumea aurita (L.F) DC	Asteraceae	Faux tabac	Leaf oil	Benin distilled
Capsicum frutescens L.	Solanaceae	Pepper	Slurry	Benin distilled
Carica papaya L.	Caricaceae	Papaya	Slurry	Benin distilled
Clausena anisata (Willd.) Hook ex f. Benth.	Rutaceae		Leaf oil	- Benin distilled - Benin bought
Combretum micranthum G. Don	Combretaceae		Slurry	Benin distilled
Cymbopogon citratus (DC. ex Nees) Stapf	Poaceae	Lemongrass	Leaf oil	Benin distilled
Mixture of *Cymbopogon citratus* & *C. flexuosus* Stapf & J. F. Watson[2]	Poaceae	Lemongrass & east Indian lemongrass	Leaf oil	Benin
Cymbopogon nardus (L.) Rendle	Poaceae	Citronella grass	Leaf oil	Togo
Cymbopogon schoenanthus (L.) Sprengel	Poaceae	Camel grass	Leaf oil	Togo
Dracaena arborea (Wild.) Link	Liliaceae	Dragontree	Slurry	Benin Mono distilled
Helianthus annuus L.	Asteraceae	Sunflower	Seed oil	The Netherlands
Hyptis spicigera Lam.	Lamiaceae	Marubio	Leaf oil	Benin distilled
Momordica charantia L.	Cucurbitaceae	Bittergourd	Slurry	Benin distilled
Nicotiana tabacum L.	Solanaceae	Tobacco	Leaf oil	Tanzania distilled
Ocimum basilicum L.	Lamiaceae	Sweet basil	Leaf oil	- Togo - Benin
Securidaca longepedunculata Fres.	Polygalaceae	Violet tree	Slurry	Benin Borgou distilled
Tagetes minuta L	Asteraceae	Mexican marigold	Leaf oil	Tanzania distilled
Tephrosia vogelii Hook f.	Leguminosae-Papilionoideae	Vogel's tephrosia	Slurry	Tanzania distilled

1: The country (and the region) mentioned is the origin of the plant material that was distilled or of the extract itself.

2: Will further be referred to as *Cymbopogon* MIX

assembled in the reservoir. After six hours, the distillation was stopped and the oil or the slurry, the clouding material, was collected with as little water as possible and stored at 4 °C in closed vials.

The extracted material of *Azadirachta indica* originated from the Zou region, that of *Dracaena arborea* from the Mono region and that of *Securidaca longepedunculata* from the Borgou region in Benin. Of these plants, several samples from different regions in Benin were tested as powders (Chapter 3) but the extracts were obtained from the samples with the most pronounced effect on *Callosobruchus maculatus*.

For *Cymbopogon nardus, Cymbopogon schoenanthus* and *Ocimum basilicum* sample 'Togo', volatile oils obtained via a procedure comparable to ours, were kindly provided by Dr. G. Ketoh, Université du Bénin, Togo. Samples of oils of *Clausena anisata* 'bought', *Cymbopogon* MIX and *Ocimum basilicum* 'Benin' were purchased in Benin and had been obtained through commercial distillation outside that country. Two non-volatile oils (*Azadirachta indica* and *Helianthus annuus*) were purchased in supermarkets in Benin and the Netherlands respectively. These oils are known to have an influence on *Callosobruchus maculatus* behaviour and development (Gupta, 1989; Kachare *et al.*, 1994; Sangappa, 1977) and were used as reference oils to help evaluate our testing methods.

Bioassays

All bioassays were done under the same conditions as the beetle rearing: 30 ± 1 °C, 50-80 % r.h. and a 12 hour photoperiod.

Toxicity

The experimental units were petri dishes with small ridges on the inside of the lid filled with 40 g (= about 200 seeds) clean uninfested cowpea beans. To prepare the treatments, 20 µl volatile oil, 200 µl non-volatile oil or 100 µl slurry were filled up to 1 ml with 96 % ethanol. These solutions were mixed with the beans through thorough shaking. Controls were treated with 1 ml 96% ethanol. The ethanol was left to evaporate for 10 minutes with the lid off the petri dish and for 5 hours with the lid on.

After evaporation of the solvent one unmated female and two male beetles were released in each petri dish on the treated or untreated beans. After 24 hours, the number of eggs was counted. The beetles were monitored daily to register their longevity. After the death of the adults, the total number of eggs was counted giving the lifetime fecundity of the female. The emerging F1 adults were sexed and removed daily. The tests were repeated 5 times per extract.

Batches of two to five plant extracts were tested at a time. With each batch, a series of five control dishes was monitored simultaneously. In total seven batches and thus seven control series were used to test all plant extracts. If the control series did not show differences

among each other, the treatments were compared with the mean values of all controls and with the values of other treatments.

Repellence

In a 75-cm glass-tube olfactometer as used in chapter 3 a jar was placed at either end of the tube. One of the jars contained 2-g beans sprayed with 20 µl 96 % ethanol as a control, the other contained treated beans. The treatments consisted of either 1 µl volatile oil, 10 µl non-volatile oil or 5 µl slurry filled up to 20 µl with 96 % ethanol sprayed on the beans without further mixing.

One freshly mated female beetle was released through a hole in the middle of the tube. The beetle was observed during its movement to the treated beans or to the control. Observations were done constantly for the first hour after release and afterwards the position in the olfactometer was noted at least once an hour. Once a beetle had entered one of the jars, it did not leave the beans anymore. After six hours, the beetles were removed from the beans. The eggs on the beans were counted one week after the experiment. Eggs had then hatched and were less vulnerable upon abrasion. For comparison with the toxicity test, the beans with eggs were incubated in petri dishes to count the number of emerging adults.

To evaluate the results, a distinction was made between beetles that actually entered one of the jars containing treated or untreated beans (a), those that did reach one end (the last five cm) of the tube (b) and those that went to one side of the tube without reaching the end (c). Only for beetles that did enter one of the jars, the eggs were counted and incubated.

The tests were replicated 36 times per plant extract in two days with a new beetle and new beans for each repetition. The second testing day the sides of control and treated beans were reversed. One plant extract, randomly picked from the list was tested per day. After each experiment, the olfactometer tubes and the jars were cleaned thoroughly.

Statistics

For the toxicity tests, a one-way ANOVA was used followed by a Bonferroni post-hoc test. For the repellence tests, a binomial test was used to investigate whether the beetles' distribution was different from 50:50. For the number of eggs, offspring and the percentage mortality in the repellence tests a Mann-Whitney U test was performed.

Results

Essential volatile oils were obtained from *Blumea aurita*, *Clausena anisata* 'distilled', *Cymbopogon citratus*, *Hyptis spicigera*, *Nicotiana tabacum* and *Tagetes minuta*. From the other plants, *Azadirachta indica*, *Capsicum frutescens*, *Carica papaya*, *Combretum micranthum*, *Dracaena arborea*, *Momordica charantia*, *Securidaca longepedunculata* and *Tephrosia vogelii* whitish slurries, mixing with water and ethanol, indissoluble in pentane were obtained.

Table 2: Life history parameters for *Callosobruchus maculatus* on treated beans in a no-choice situation (means of five repetitions per treatment and 7 series of 5 controls ± S.D). Numbers in columns followed by the same letter are not significantly different (P > 0.05).

Treatment	Eggs after 24h	Longevity		Total eggs	Number of beetles emerged	Percentage mortality
		Females	Males			
Control	15.1 ± 9.5 ab	8.3 ± 1.9 a	8.7 ± 1.4 a	91.9 ± 24.0 a	67.6 ± 19.9 a	28.6 ± 15.4 a
Azadirachta indica oil	12.0 ± 5.5 ab	8.2 ± 0.8 a	6.6 ± 0.7 ab	84.6 ± 28.7 ab	0.8 ± 0.5 bc	98.9 ± 0.8 bcd
Azadirachta indica slurry	16.6 ± 5.1 ab	9.2 ± 2.2 a	9.9 ± 0.2 a	96.6 ± 19.4 a	67.4 ± 26.5 ab	32.2 ± 16.4 ac
Blumea aurita	13.4 ± 7.5 ab	10.2 ± 2.9 a	8.7 ± 0.9 ab	122.0 ± 32.8 a	87.4 ± 20.5 a	27.6 ± 7.0 a
Capsicum frutescens	24.2 ± 9.5 b	8.2 ± 0.4 a	9.6 ± 1.4 a	111.8 ± 27.0 a	87.6 ± 21.2 a	21.1 ± 8.4 a
Carica papaya	26.4 ± 15.8 b	8.2 ± 1.9 a	8.7 ± 2.0 ab	79.8 ± 15.7 abc	55.0 ± 16.8 ab	32.4 ± 8.8 ac
Clausena anisata distilled	14.3 ± 5.4 ab	10.3 ± 1.7 a	9.1 ± 1.5 a	115.0 ± 17.6 a	82.8 ± 12.9 a	27.8 ± 4.6 a
Clausena anisata bought	10.6 ± 11.1 ab	8.8 ± 1.3 a	8.9 ± 0.7 ab	71.0 ± 29.8 abc	51.8 ± 20.7 ab	25.4 ± 8.8 a
Combretum micranthum	21.8 ± 5.1 ab	9.2 ± 2.2 a	8.1 ± 1.4 ab	94.2 ± 27.2 a	66.0 ± 16.4 ab	28.6 ± 7.8 a
Cymbopogon citratus	7.0 ± 9.9 ab	7.2 ± 1.3 a	7.2 ± 1.2 ab	69.0 ± 15.7 b	53.6 ± 17.4 ab	23.6 ± 10.6 a
Cymbopogon MIX	7.6 ± 6.2 ab	8.8 ± 0.8 a	8.4 ± 1.0 ab	72.4 ± 18.5 abc	51.8 ± 12.2 ab	27.8 ± 7.5 a
Cymbopogon nardus	0.0 ± 0.0 a	8.2 ± 0.8 a	7.0 ± 0.8 ab	26.8 ± 39.3 bc	19.4 ± 30.3 bc	53.1 ± 41.4 bcd
Cymbopogon schoenanthus	0.6 ± 0.9 a	7.0 ± 2.5 a	5.3 ± 1.6 b	0.8 ± 0.8 bc	0.0 ± 0.0 bc	100.0 ± 0.0 bcd
Dracaena arborea	16.9 ± 11.6 ab	9.6 ± 1.5 a	8.9 ± 1.1 ab	99.6 ± 15.5 a	66.8 ± 26.7 ab	34.6 ± 22.8 ac
Helianthus annuus	9.4 ± 7.1 ab	7.0 ± 2.1 a	6.6 ± 0.7 ab	69.0 ± 33.3 ab	22.4 ± 12.9 bc	66.7 ± 9.1 bcd
Momordica charantia	12.0 ± 11.6 ab	8.3 ± 1.2 a	9.3 ± 0.9 ab	94.0 ± 29.0 a	68.6 ± 21.4 a	26.9 ± 6.1 a
Nicotiana tabacum	9.2 ± 9.3 ab	9.2 ± 2.8 a	9.4 ± 1.2 a	89.0 ± 38.7 a	63.6 ± 31.0 ab	28.4 ± 13.6 a
Ocimum basilicum Togo	6.4 ± 10.1 ab	9.2 ± 3.6 a	7.9 ± 2.8 ab	25.0 ± 33.4 abc	18.8 ± 25.8 bc	49.0 ± 44.2 abc
Ocimum basilicum Benin	5.6 ± 5.5 ab	8.6 ± 0.9 a	9.0 ± 0.7 a	75.4 ± 26.0 abc	59.4 ± 23.1 ab	22.0 ± 5.1 a
S. longepedunculata	19.3 ± 12.5 ab	9.2 ± 1.6 a	9.4 ± 1.1 a	97.4 ± 25.4 a	79.4 ± 22.9 a	18.7 ± 6.1 a
Tagetes minuta	10.2 ± 8.9 ab	10.2 ± 2.3 a	9.9 ± 1.3 a	96.8 ± 13.0 a	54.4 ± 17.8 ab	43.4 ± 17.1 abc
Tephrosia vogelii	24.6 ± 9.8 b	9.2 ± 1.8 a	9.0 ± 0.8 a	96.0 ± 24.0 a	65.4 ± 17.1 ab	31.8 ± 6.4 ac

Toxicity

The results of the toxicity test are shown in Table 2. There were no differences between the control batches for any parameter. We therefore compared all treatments with the mean of all controls and among each other.

The number of eggs after 24 hours was not different from the control for any of the treatments. The beans treated with *Cymbopogon nardus* or *Cymbopogon schoenanthus* received fewer eggs than beans treated with *Capsicum frutescens*, *Carica papaya* or *Tephrosia vogelii*.

The longevity of the female parent beetle was not altered by any of the treatments and the treatments did not differ from each other. Male beetles died earlier on beans treated with *Cymbopogon schoenanthus* than on the control beans or on beans treated with slurries of *Azadirachta indica*, *Capsicum frutescens*, *Securidaca longepedunculata* or *Tephrosia vogelii* or with oil of *Clausena anisata* 'distilled', *Nicotiana tabacum*, *Ocimum basilicum* 'Benin' or *Tagetes minuta*.

The total number of eggs laid during the life of the female beetle was lowered by the treatment with *Cymbopogon citratus* and even more so by *Cymbopogon nardus* and *Cymbopogon schoenanthus*.

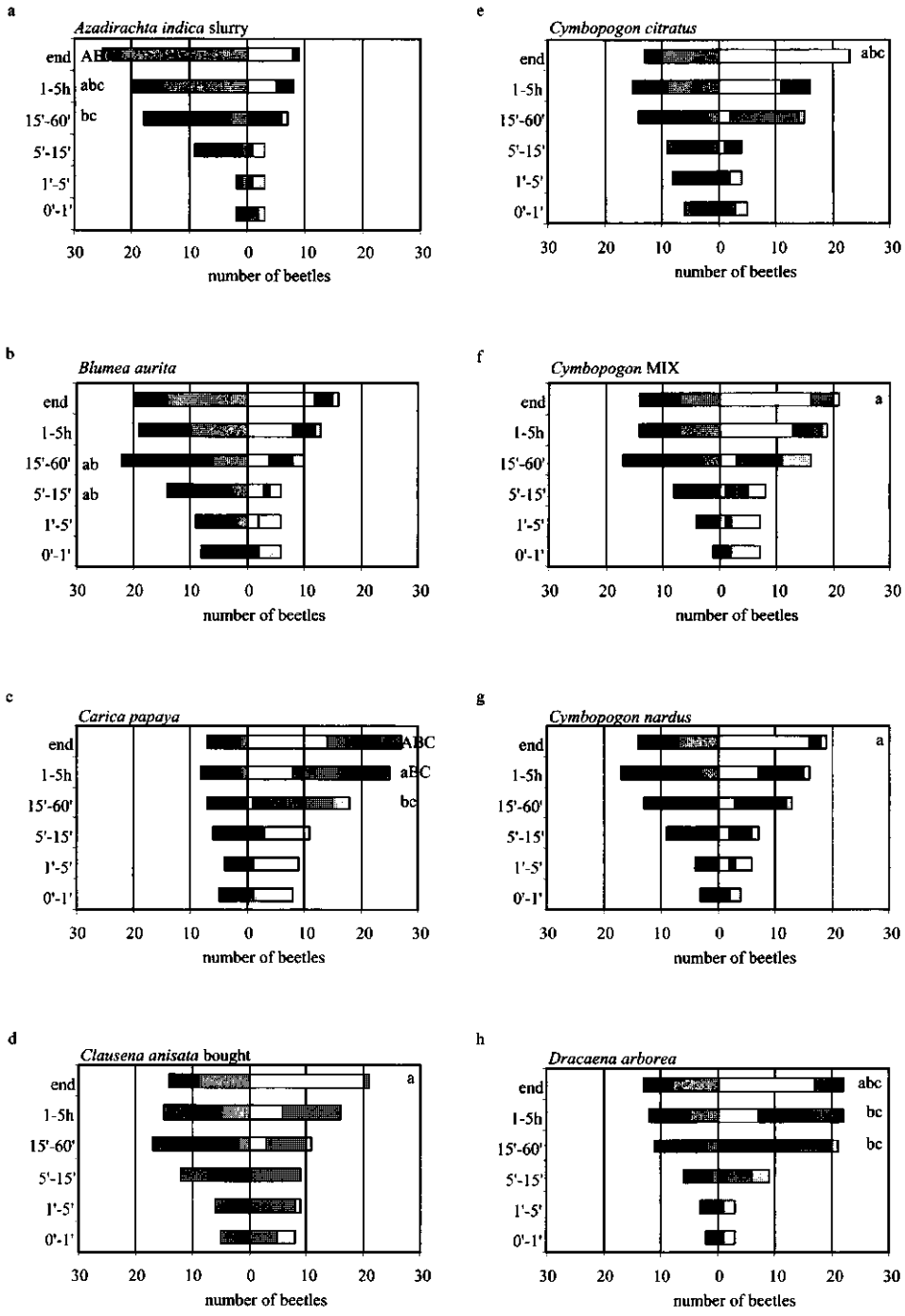
The number of beetles emerging from untreated beans was higher than in batches treated with *Ocimum basilicum* 'Togo', *Cymbopogon nardus*, *Cymbopogon schoenanthus* and with the non-volatile oils of *Azadirachta indica* and *Helianthus annuus*. For the last four of these, the percentage mortality of immature stages of the beetle, as eggs, larvae or pupae was higher than on control beans.

Repellence

No repellent or attractive effects were recorded for both non-volatile oils *Azadirachta indica* and *Helianthus annuus*, the slurries of *Capsicum frutescens*, *Combretum micranthum*, *Momordica charantia* and *Securidaca longepedunculata* and for the volatile oils extracted from *Clausena anisata* 'distilled', *Cymbopogon schoenanthus* and *Nicotiana tabacum*. The results for extracts evoking a significant effect at least at one point during the experiment are shown in Figure 1.

Two extracts had an attractive effect on the beetles. The slurry of *Blumea aurita* attracted beetles to the treatment side and end of the tube during the experiment, but at the end of the experiment, no effect was measured. For beans treated with the slurry distilled from *Azadirachta indica* leaves, more beetles went to the treatment side and end of the tube and more entered the treatment jars.

The other extracts had a repellent effect at least at some point during the experiment. The oil of *Ocimum basilicum* 'Togo' made the beetles go to the control side of the tube immediately and at the end of the experiment many more beetles had entered the control jar. The oils of *Cymbopogon citratus* and *Hyptis spicigera* and the slurries of *Carica papaya*, *Dracaena*


a
Azadirachta indica slurry
end
1-5h
15'-60'
5'-15'
1'-5'
0'-1'
AB
abc
bc
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number of beetles
b
Blumea aurita
ab
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c
Carica papaya
ABC
aBC
bc
d
Clausena anisata bought
a
e
Cymbopogon citratus
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Cymbopogon MIX
a
g
Cymbopogon nardus
a
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Dracaena arborea
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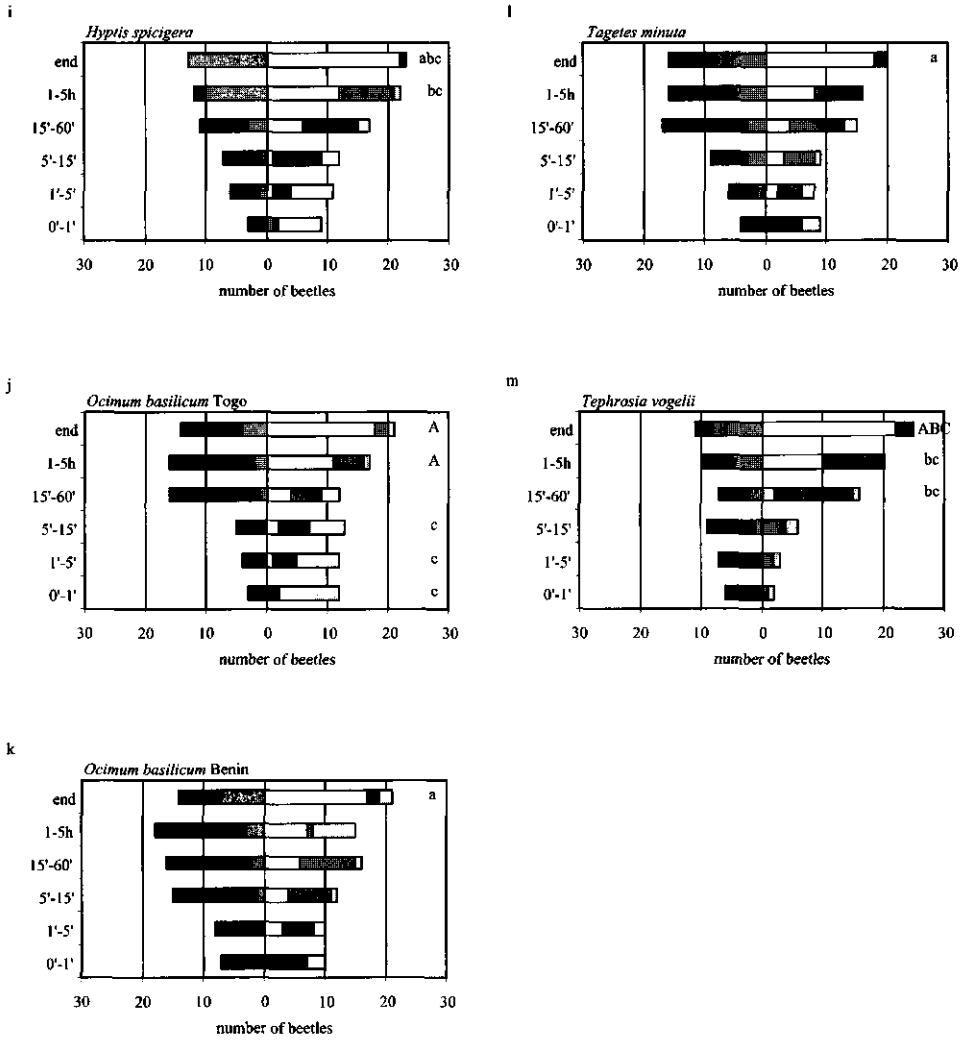

Figure 1 a-m: Distribution of *Callosobruchus maculatus* beetles in a linear olfactometer: Numbers of beetles = in the jar with treated beans, = at the treatment end of the olfactometer, = at the treatment side, = in the jar with untreated control beans, = at the control end, = at the control side. Data on beetles that did not make a choice are not incorporated in the figure. a (& A) = more beetles in the jar with beans at this side of the olfactometer tube P< 0.05 (& P< 0.01), b (&B) = more beetles at this end of the tube P< 0.05 (& P< 0.01), c (&C) = more beetles at this side of the tube P< 0.05 (&P < 0.01).

arborea and *Tephrosia vogelii* made the beetles go to the control side and end of the tube and more beetles had entered the control jars at the end of the experiment. The slurry of *Tagetes minuta* and the oils of *Clausena anisata* 'bought', *Cymbopogon* MIX, *Cymbopogon nardus* and *Ocimum basilicum* 'Benin' made more beetles enter the control jars.

For the beetles that entered a jar and had the possibility to lay viable eggs, the numbers of eggs and of emerging offspring per beetle are shown in Table 3. For the non-volatile oil of *Azadirachta indica* more eggs were laid on control beans and more offspring emerged from these. The percentage mortality of developing beetles was higher on treated beans. For the other non-volatile oil, *Helianthus annuus*, only the percentage mortality was higher for treated beans, but the oviposition behaviour of the beetle was not affected. The volatile oils of both samples of *Clausena anisata*, the oils of all tested *Cymbopogon* species and *Ocimum basilicum* 'Togo' caused lower numbers of eggs to be laid on treated beans and thus a lower number of offspring to emerge. No eggs were laid on beans treated with *Cymbopogon* MIX. The oils of *Cymbopogon citratus*, *Cymbopogon nardus* and *Ocimum basilicum* 'Togo' caused an increase of the percentage of mortality of immature stages of the beetle. For the slurry of *Carica papaya*, no comparison could be made since only a single beetle entered a jar with treated beans.

Discussion

Non-volatile oils, especially that of *Azadirachta indica*, were very effective in blocking the development of eggs laid on treated beans. The non-volatile oils had no repellent effect, the adult beetles lived just as long as on untreated beans and the numbers of eggs laid were not lower. The numbers of offspring were low on beans treated with *Helianthus annuus* oil, and on beans treated with *A. indica* even fewer beetles emerged than were introduced initially. Jadhav and Jadhav (1983) found the same trends for *A. indica* oil applied at 3 ml/kg, but at 10 ml/kg Naik and Dumbre (1985) found a repellent or at least oviposition repellent effect. They showed that if the beetles were given a direct choice and the possibility to leave the chosen patch, beetles laid fewer eggs on beans treated with *A. indica* oil than on untreated beans. For *H. annuus* oil, our results are comparable to those of Shaaya and Ikan (1981) although these authors used a lower dose of oil.

If the volatile oils had an effect on the beetles' life history, it was mostly through the inhibition of oviposition and the consequently low numbers of emerging beetles. The percentage of pre-imaginal mortality was higher than on untreated beans after treatment with *Cymbopogon nardus* and *C. schoenanthus*. A direct effect on adult beetles was found as the shorter longevity of male beetles on beans treated with *Cymbopogon schoenanthus*. Likewise, Glitho *et al.* (1997) and Ketoh *et al.*, (2000) found that the effect on the longevity of adult bruchids was significant for *C. schoenanthus*, but not for *C. citratus* and *C. nardus*.

Table 3: Life history parameters for *Callosobruchus maculatus* on treated beans in a two-choice situation. The number of eggs and offspring are mean values per beetle for the number of beetles that entered a jar at a certain side of the olfactometer. * : $P < 0.05$, **: $P < 0.01$ for binomial 50:50 distribution (number of beetles) or Mann Whitney U test (eggs, offspring, mortality; ± S.D.), -- : not calculated.

Treatment		Beetles in jar			Number of eggs			Number of offspring			Percentage mortality		
	ml/kg	C	T		C	T		C	T		C	T	
Azadirachta indica oil	5.0	14	10		17.6 ± 6.5	3.7 ± 3.1	**	15.3 ± 6.1	0.6 ± 0.7	**	13.0 ± 10.3	88.1 ± 17.3	**
Azadirachta indica slurry	2.5	8	22	**	17.8 ± 6.6	13.3 ± 11.0		16.8 ± 6.0	11.5 ± 9.4		6.1 ± 4.1	11.5 ± 13.4	
Blumea aurita	0.5	12	14		12.6 ± 7.3	7.3 ± 7.0		11.1 ± 6.7	6.9 ± 6.7		12.6 ± 12.9	12.2 ± 29.5	
Capsicum frutescens	2.5	11	17		13.2 ± 9.6	16.6 ± 9.7		11.1 ± 8.3	14.3 ± 8.7		16.4 ± 13.6	14.7 ± 11.9	
Carica papaya	2.5	14	1	**	5.7 ± 6.7	6		4.4 ± 5.6	6		26.4 ± 22.5	0	
Clausena anisata distilled	0.5	18	13		18.3 ± 5.7	5.5 ± 8.2	**	15.9 ± 5.1	4.4 ± 6.7	**	12.8 ± 10.2	31.8 ± 34.4	
Clausena anisata bought	0.5	20	9	*	14.7 ± 7.3	2.3 ± 3.6	**	12.2 ± 6.2	2.1 ± 3.1	**	15.2 ± 14.0	4.7 ± 6.5	
Combretum micranthum	2.5	10	13		16.4 ± 13.9	14.2 ± 7.2		14.0 ± 12.9	12.1 ± 7.3		22.0 ± 32.4	15.7 ± 12.4	
Cymbopogon citratus	0.5	23	10	*	15.7 ± 7.3	4.1 ± 8.1	**	13.9 ± 7.0	3.1 ± 7.5	**	13.0 ± 11.0	59.7 ± 45.4	**
Cymbopogon MIX	0.5	16	7	*	10.3 ± 6.9	0.0 ± 0.0	**	9.4 ± 6.6	0.0 ± 0.0	**	14.8 ± 25.2	--	--
Cymbopogon nardus	0.5	16	7	*	16.8 ± 6.9	3.5 ± 3.6	**	14.1 ± 5.8	0.9 ± 2.5	**	16.3 ± 13.4	80.0 ± 44.7	*
Cymbopogon schoenanthus	0.5	16	11		12.1 ± 5.9	1.4 ± 3.3	**	10.4 ± 5.2	1.0 ± 3.3	**	14.5 ± 11.0	75.0 ± 50.0	
Dracaena arborea	2.5	17	8	*	12.6 ± 7.0	14.5 ± 7.7		11.6 ± 6.8	13.5 ± 7.1		9.2 ± 9.2	10.4 ± 17.1	
Helianthus annuus	5.0	15	13		10.0 ± 8.2	11.8 ± 5.6		9.3 ± 7.5	7.8 ± 4.7		4.8 ± 5.8	39.0 ± 23.4	**
Hyptis spicigera	0.5	22	13	*	17.5 ± 7.6	18.3 ± 7.0		15.0 ± 7.4	14.5 ± 4.9		14.0 ± 18.7	18.5 ± 13.0	
Momordica charantia	2.5	14	16		12.3 ± 8.6	13.1 ± 8.3		9.1 ± 7.2	11.3 ± 8.0		15.5 ± 15.5	14.5 ± 25.8	
Nicotiana tabacum	0.5	13	7		12.8 ± 10.4	4.4 ± 4.7		11.4 ± 9.0	3.7 ± 4.4		9.3 ± 6.1	20.3 ± 20.7	
Ocimum basilicum Togo	0.5	18	4	**	13.0 ± 7.8	2.0 ± 2.8	*	10.9 ± 6.9	1.8 ± 2.4	*	20.0 ± 22.3	8.3 ± 11.8	
Ocimum basilicum Benin	0.5	17	7	*	10.5 ± 8.5	4.9 ± 6.8		9.5 ± 8.1	4.7 ± 6.7		13.9 ± 14.1	3.0 ± 5.3	
S. longepedunculata	2.5	14	11		11.9 ± 6.9	12.1 ± 7.6		9.5 ± 5.6	10.2 ± 7.4		17.3 ± 19.9	16.3 ± 20.4	
Tagetes minuta	0.5	18	8	*	12.9 ± 11.2	9.8 ± 8.2		11.3 ± 10.3	8.5 ± 7.6		15.0 ± 8.5	20.5 ± 35.7	
Tephrosia vogelii	2.5	22	8	**	14.1 ± 9.3	11.4 ± 4.1		12.7 ± 8.0	10.9 ± 4.4		8.0 ± 9.6	7.2 ± 12.1	

However, these authors reported that the reduction of the number of eggs laid by the beetles after treatment of the beans with *C. citratus* was more important than the reduction caused by *C. nardus*, whereas we found the opposite.

The dosage of volatile oil we applied, i.e. 0.5 ml/kg, is low but not exceptionally low compared to data from the literature (Part I of this thesis). When applied at doses of 1.25 or even 2.50 ml/kg the oils of *Clausena anisata*, *Cymbopogon* MIX, *Cymbopogon nardus* and *Cymbopogon schoenanthus* prevented oviposition completely and reduced the longevity of the adult beetles drastically. Oils of the two samples of *Ocimum basilicum* were only slightly less effective, but here as well, the development of an F1 generation of beetles was completely prevented at these high doses. However, if the same beans were reinfested fifteen days later, the effect of the oils was not noticeable anymore (Barnaud, unpublished data).

Due to a lack of plant material, the oil of *Hyptis spicigera* could not be tested for its toxic effect against the beetle. There is reason to believe the oil would not have much effect. When applied topically, this oil was found to have a toxic effect on adult *C. maculatus* beetles with an LD_{50} of 0.142-0.257 µl/ insect (Anonymous, 1994), but it was less effective than oil of *Ocimum basilicum* (Djibo *et al.*, 1996).

All plants that were extracted here, were selected because their leaf powder had shown at least some effect on the development or the behaviour of *Callosobruchus maculatus* (Chapter 3). For the plants from which no volatile oil was obtained, the extracts did not show any toxic effect on the beetles. The leaf powder of *Tephrosia vogelii* caused adult beetles to die before they could lay a large number of eggs and the eggs that were laid mostly did not develop into adult beetles. However, the slurry extracted from the powder did not affect the life history of the beetle at all. The lower number of eggs laid after 24 hours on beans treated with powders of *Azadirachta indica*, *Capsicum frutescens* or *Combretum micranthum* and the high percentage mortality of immature stages on beans treated with powder of *Securidaca longepedunculata* (Chapter 3) were not found on beans treated with the slurries of these plants.

The cause of the lack of insecticidal effect of the slurries is not known. The active compounds could be so volatile that, as soon as the plant cells are ruptured and heated, the compounds escape. They could also be heat sensitive and therefore have degraded at the high temperatures in the distillation set up. Another option is that the active ingredients are still in the plant material that was discarded after the distillation process. Or the combination of ingredients that caused the effect of the crude plant material could have been separated, with one part still in the plant material and the other part(s) evaporated or present in the slurry.

Not all activity had disappeared from the slurries however. The slurries of *Carica papaya*, *Dracaena arborea* and *Tephrosia* had repellent effects, whereas the slurry of *Azadirachta indica* was attractive. This attractive effect was also found for the slurry of *Blumea aurita*,

which is the complete opposite of the strong repellent activity of the powder of the same plant.
The quantity of slurry applied in the repellence test is more or less arbitrary. The material was obtained in water and the concentrations and chemical composition of these mixtures have not been determined. All slurries had pungent smells.
The period between the distillation and the actual test varied from 24 hours to a few weeks. If there would have been an effect with time on the plant extracts, the results of the repellence tests might have been different for the two test days per treatment. When the end-results of both test days were compared (R x C Test of independence, Sokal and Rohlf, 1995), differences were found for *Blumea aurita*, *Clausena anisata* 'bought' and *Securidaca longepedunculata*. Volatile oils are usually preserved well under the storage conditions we employed: in closed vials at 4°C in the dark. The oil of *Clausena anisata* 'bought' had been distilled at least six months before the time of the tests. We presume that the short time span between the two tests did not have a major influence on the composition of the extracts. The differences we found might be due to the biological variation in the beans or the beetles.

Developmental data from the toxicity and repellence tests were not pooled because the eggs in the repellence tests were laid only during the first day after emergence of the female. Such early laid eggs have a better chance of survival than eggs laid later during the life of the adult beetle (Nwanze & Horber, 1975). Support for this statement was found when the percentages mortality were compared for treated beans in the two tests (toxicity and repellence) with a Mann Whitney-U test. For the treatments where these percentages were different (*Azadirachta indica* slurry, *Blumea aurita*, *Clausena anisata* 'bought', *Combretum micranthum*, *Dracaena arborea*, *Helianthus annuus*, *Momordica charantia*, *Ocimum basilicum* Benin and *Tephrosia vogelii*) the percentages mortality in the toxicity test with eggs laid during the whole lifetime of the parents, were always higher than for the repellence tests with oviposition only during the first day of the beetles' life. This better chance of successful development of beetle eggs in the repellence test might also be due to the fact that in this test, the plant extracts were not thoroughly mixed with the beans, so the beetles might have laid their eggs on untreated surfaces.
On the other hand, the beans were treated shortly prior to the actual experiment and thus, at the end of the repellence tests the treatments had lasted only little more than six hours, whereas in the toxicity tests the plant products were on the beans for at least a week. Moreover, only two grams of beans, nine seeds were offered for oviposition in the repellence test. Mean numbers of eggs laid on these seeds were as high as 18 which indicates that more than one larva had to develop per bean, which could have had a negative influence on their survival (Hu *et al.*, 1995; Ofuya, 1997a). In the toxicity tests, 200 seeds were offered per female, which would give the beetle the opportunity to lay all eggs on separate beans.

We conclude that the slurries of *Carica papaya*, *Dracaena arborea* and *Tephrosia vogelii* have a strong repellent effect. The toxic effect of the powders of these plants was lost in the process of extraction. Non-volatile oils do not prevent oviposition on beans, but the pre-imagial development is negatively affected. The effect of such oils usually lasts for months, but the effects on the stored beans themselves are numerous as well (Part I of this thesis). Volatile oils, especially if they are applied in high enough quantities, are repellent and toxic to the beetle. However, such oils loose their efficacy within two weeks even in the relatively closed environment of a petri dish. Since the initial beetle infestation mostly takes place in the field and is brought into the storage facility with freshly harvested beans (Prevett, 1961), oils could serve as reducers or blocking agents for this first infestation. Re-infestation, from alternative host plants or other stores which is usually less important, should be prevented.

Acknowledgements

The research was financed by the Centre Béninois pour le Développement Durable. We thank Dr. G. Ketoh for providing volatile oils of *Cymbopogon nardus*, *C. schoenanthus* and *Ocimum basilicum*. Mr G. Lelyveld of the Laboratory of Organic Chemistry of Wageningen University is acknowledged for his help with the extractions.

CHAPTER 5

SIDE EFFECTS OF COWPEA TREATMENT WITH BOTANICAL INSECTICIDES ON TWO PARASITOIDS OF *CALLOSOBRUCHUS MACULATUS*

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Abstract

Studies on the protective effect of botanical products against pest insects have hardly been extended to side effects on natural enemies. Indirect effects of the botanicals on the storability of seeds could occur through their possible negative impact on biological control agents. Four plant powders and six plant oils with a known effect on the cowpea beetle *Callosobruchus maculatus* were investigated for their effects on the beetles' egg parasitoid *Uscana lariophaga* and the larval parasitoid *Dinarmus basalis*. All treatments caused a decrease in parasitisation by *U. lariophaga* and developing parasitoids were affected by powders of *Nicotiana tabacum* and *Tephrosia vogelii*. In a two-choice situation in a linear olfactometer, *U. lariophaga* was repelled by most of the oils. In a no-choice situation, oviposition by *D. basalis* was hampered by treatment with plant powders, but the eggs that were laid developed normally. In a Y-tube olfactometer, this parasitoid did not discriminate between odours of untreated and plant-powder-treated beans. The powders of *N. tabacum* and *T. vogelii* had stronger negative effects on the two parasitoids than the powders of *Azadirachta indica* and *Blumea aurita*. In untreated samples collected from traditional storage facilities and treated with plant powders in the laboratory, none of the treatments could prevent the build-up of a beetle population. At 24 days after treatment, most beetles had emerged from beans treated with powders of *N. tabacum* and *T. vogelii*. Parasitoids were affected by the botanical insecticides tested here, but powders of *A. indica* and *B. aurita* may be compatible with biological control by *D. basalis*.

Key words: seed storage, *Uscana lariophaga*, *Dinarmus basalis*, plant powders, volatile oil, non-volatile oil

Introduction

To protect stored cowpea beans (*Vigna unguiculata* (L.) Walp) against their most important insect pest, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) many products have been tested. If no treatment is applied during storage, all beans will be attacked and damaged by the beetle within six months (Singh, 1977). However, in the presence of parasitoid wasps that parasitise the developing beetles, the percentage of damaged seeds will not exceed 60 % (Caswell, 1973). Percentages of parasitism on *C. maculatus* in storage structures in West Africa were as high as 73 % for bruchid eggs and 89 % for the larvae (Van Alebeek, 1996).
Cowpea is a widely spread crop of tropical areas and it is often cultivated by small-scale farmers in Africa. Keeping these growers in mind when searching for a means to protect stored cowpea, the use of insecticides derived from plant products available on the spot has been investigated. Nowadays much research is done to revert to these less expensive, safer, and better available traditional practices (reviewed in Part I of this thesis).
However, if these plant products are effective against *C. maculatus*, potential negative effects on other insects (Sanon *et al.*, 2002) should be considered before these products are applied. The beetles' development mostly takes place inside the bean, whereas natural enemies such as egg-parasitoids spend their whole life and larval parasitoids spend part of their life cycle on the bean surface in contact with the botanical (Van Alebeek, 1996). Since the wasps are important for suppressing the beetles' population, it is useful to investigate the effect of botanical insecticides on two major parasitoids of *C. maculatus*: the egg parasitoid *Uscana lariophaga* and the larval parasitoid *Dinarmus basalis*.

Material and methods

All tests, incubations and rearings were done at 30 ± 2°C, 60 ± 5 % r. h. with a 12:12 photo: scotophase.

Beetles

Callosobruchus maculatus (Fabricius) (Coleoptera: Bruchidae) was reared on cowpea seeds. To obtain fourth instar larvae, beetles were allowed to oviposit on uninfested cowpeas during 24 hours. After 16 days, the eggs had developed into fourth instar larvae.

Parasitoids

The egg parasitoid *Uscana lariophaga* (Steffan) (Hymenoptera: Trichogrammatidae) was reared on fresh eggs of *C. maculatus* on cowpea seeds. The larval parasitoid *Dinarmus basalis* (Rondani) (Hymenoptera: Pteromalidae) was reared in glass jars on fourth instar larvae of *C. maculatus* in cowpea beans. For all tests, newly emerged adult parasitoids were used.

Plant materials

Four plant powders and six plant oils, of which the effects against *C. maculatus* have previously been reported (Chapter 3 and 4), were used in this study (Table 1). For the powders, fresh leaf samples were collected in Benin (West Africa) and dried there at ambient temperature in a shaded place. Leaves were ground in a wooden mortar and the powders were sieved before use. Plant powders were always applied at 25 g/kg beans.

The plant oils were purchased in West Africa in Togo and Benin. The volatile oils of Rutaceae, Poaceae and Lamiaceae, applied at 0.5 ml/kg beans, all had potent effects on the beetles (Chapter 4). The non-volatile neem oil, applied at 5 ml/kg beans, prevented the emergence of a new beetle generation through mortality of immature stages of the beetle (Chapter 4).

Table 1: Tested plant materials and their formerly found effects (Chapter 3 and 4) on *Callosobruchus maculatus*

Plant	Plant family	Common name	Effect
Powders			
Azadirachta indica (Juss.)	Meliaceae	Neem	Attractive
Blumea aurita (DC)	Asteraceae	False tobacco	Repellent
Nicotiana tabacum (L.)	Solanaceae	Tobacco	Toxic (ovicidal)
Tephrosia vogelii (Hooker f.)	Leguminosae Papilionideae	Vogel's tephrosia	Toxic and repellent
Oils			
Azadirachta indica (Juss.)	Meliaceae	Neem	Toxic
Clausena anisata ((Willd.) Hook ex f. Benth.	Rutaceae	Clausena	Repellent
Mixture of *Cymbopogon citratus* (DC. ex Nees) Stapf and *C. flexuosus* (Nees ex Steudel) J. F. Watson	Poaceae	*	Repellent
Cymbopogon nardus (L.) Rendle	Poaceae	Citronella grass	Toxic and repellent
Cymbopogon schoenanthus (L.) Sprengel	Poaceae	Camel grass	Toxic
Ocimum basilicum (L.)	Lamiaceae	Basil	Repellent

*: Here referred to as *Cymbopogon* MIX

Effect on *Callosobruchus maculatus*

The effects of the powders on *C. maculatus* have been tested before (Chapter 3). However, since biological variation between samples of leaves may occur, we decided to test the effect of the newly obtained plant powders on the beetle as well. To petri dishes with 20 g of

uninfested cowpea beans, 0.5 g plant powder was added for the treatments and the controls were left untreated. In each dish, ten pairs of newly emerged beetles were released to test the effect of the powders on oviposition. After 24 h, the beetles were removed and the number of eggs they had laid was counted.
To analyse the effect of the plant powders on the pre-imaginal stages, beetles were allowed to lay eggs during 24 h on 20 g untreated beans in petri dishes. After this period, the beetles were removed and the beans were then treated with 0.5 g plant powder. Eggs were counted five days later and the number of emerged F1 adults was recorded 24 days after oviposition. Six replicates were run for the treatments and for untreated controls. The numbers of eggs were log-transformed and the data were subjected to a one-way ANOVA and a Tukey post hoc test. For the oils the samples had been used in earlier research and the effects on the beetle had been investigated already (Chapter 4) the oils had been stored in glass bottles at 4°C in the dark.

Effects on the egg parasitoid *Uscana lariophaga*
In a petri dish with 10 g cowpea seeds, *C. maculatus* beetles were allowed to lay eggs for 24 h, after which the beetles were removed.
To test for a inhibitory effect of plant powders on oviposition and for toxicity against adult parasitoids, the beans with beetle eggs were treated with 0.25 g plant powder. Then three pairs of newly emerged *U. lariophaga* parasitoids were released on the beans. After 24 h, the number of dead parasitoids was counted. After five days, when the bruchid eggs that were parasitised had turned brown or black (Prevett, 1961) and most of the unparasitised eggs had turned whitish as a sign of the beetle larva having hatched, the parasitised eggs were counted. Six repetitions were done per treatment.
The toxic effect of plant oils on parasitoids was evaluated in a similar set-up. Beans with fresh (less than 24 h old) beetle eggs were treated with a mixture of ethanol and oil. Volatile oils were dissolved at 20 µl/ml ethanol and neem oil at 100 µl/ml. The ethanol was added to facilitate the application and proper mixing of the small quantities of oil. The solution was applied at 25 ml/kg, which resulted in the beans being treated with 0.5 ml/kg volatile oil or with 5 ml/kg non-volatile neem oil. The ethanol was allowed to evaporate for 24 h. During evaporation, the beans were placed at 4 °C so that the development of the beetle eggs would be slow and the eggs would still be accepted as a host for the parasitoids after solvent evaporation. Two treated beans with eggs were offered to a newly emerged parasitoid in a glass tube. After 24 h, the wasp was removed and the number of parasitised eggs was counted four days later. Untreated and ethanol treated beans with eggs were tested as controls. The experiments were repeated 7 to 9 times with new wasps and beans in new tubes for each replication.

To evaluate the effect of the plant powders on developing parasitoids, the fresh beetle eggs were parasitised before treatment with powders. The number of emerged parasitoids was counted. This experiment was repeated five times.
The number of eggs was log-transformed and the data were analysed in an ANOVA followed by a LSD post-hoc test.
The effect of the oils on parasitoid orientation behaviour was investigated in an I-tube olfactometer set-up consisting of a 10-cm glass tube of 0.75 cm diameter with a 4 mm hole in the middle. One treated and one untreated bean were put at either end of the tube. The tube was then closed with plastic stoppers and one parasitoid was released through the centre hole, which was then covered with parafilm. The behaviour of the wasp was observed until it reached one of the beans. Treated and control beans were alternated between sides. To reveal a possible bias in the set-up, tests with an untreated bean at either side of the olfactometer tube were performed as well. The tests were repeated ten times per treatment, using new wasps, new beans and clean tubes for every replication. The data were tested for a deviation from a binomial distribution of 50:50, which was assumed to represent no effect.

Effects on the larval parasitoid *Dinarmus basalis*

The plant powders were tested for their effects on the larval parasitoid *D. basalis*. In a petri dish containing 20 cowpea beans with beetle eggs, the number of hatched eggs of *C. maculatus* was counted and considered to represent the number of fourth instar larvae. For a test on the inhibitory effect on oviposition and adult longevity, the beans were then treated with 0.5 g plant powder and three pairs of newly emerged *D. basalis* were introduced. The adults were observed daily until their death and their life span was noted in days. The number of F1 adults emerging from the seeds was recorded.
To investigate the effect of plant powders on immature stages, the beans were offered for parasitisation before treatment with plant powders. The number of emerging parasitoids was recorded. Six replicates were done for these experiments. The numbers of eggs were log-transformed and the data were subjected to an ANOVA followed by a Tukey multiple comparison test.
A Y-tube olfactometer as described by Takabayashi and Dicke (1992) was used to evaluate the host location behaviour of *D. basalis* towards treated or untreated beans. Odour sources consisted of 200 g cowpea holding fourth instar larvae and either untreated for the control or treated with 5 g plant powder for the treatments. Two airstreams with a flow rate of 3.5 l/min were led through two separate 250 ml glass vessels containing one control and one treated odour source and then through the olfactometer arms. Individual parasitoids were introduced at the base arm of the olfactometer and exposed to odours from untreated and treated beans. Parasitoid behaviour was noted until they reached the end of an olfactometer arm or during a maximum of five minutes. Thirty insects were tested per treatment. The connection of odour source containers to the Y-tube was exchanged after five insects were

tested. The insects that had not made a choice after five minutes were excluded from the statistical analysis. The data were tested for a deviation of a binomial distribution of 50:50, which was assumed to represent no effect.

Study on seeds collected from granaries

From traditional granaries in Benin containing untreated cowpea beans with their insect fauna, a sample of 6 kg was taken to the laboratory. The sample was divided into 30 samples of 200 g in separate glass jars. These sub-samples were either treated with 5 g of one of the plant powders or left untreated as a control. The jars were covered with a muslin cloth and incubated. From each jar at 0, 8 and 24 days after treatment, a random sample of 100 seeds (± 17 g) was taken and on these seeds the numbers of parasitised and unparasitised eggs of *C. maculatus* were counted. The numbers of dead or living beetles and parasitoids were counted at each of the three time points for the whole 200 g sample after having taken the 100 seeds. Each treatment was repeated six times.

The data were log-transformed and analysed with a two-way ANOVA and a Tukey multiple comparison test.

Table 2: A) Inhibitory effects on oviposition and B) effects of plant powders on the development of *Callosobruchus maculatus* (beans treated after oviposition) (means ± S.D.). Values in columns followed by the same letter are not different ($\alpha = 0.05$).

A

	Number of eggs laid / 10 females
Control	290 ± 69.6 a
Azadirachta indica	130 ± 31.6 b
Blumea aurita	140 ± 37.4 b
Nicotiana tabacum	157 ± 55.0 b
Tephrosia vogelii	125 ± 28.8 b

B

	Number of eggs treated	Hatched eggs (%)	Emergence (%)	Emerged adults alive (%)
Control	229 ± 89.6 a	95 ± 3.9 a	71 ± 13.6 a	86 ± 16.3 a
Azadirachta indica	183 ± 64.3 a	95 ± 3.8 a	76 ± 7.7 a	83 ± 3.1 a
Blumea aurita	183 ± 96.2 a	97 ± 2.6 a	76 ± 19.1 a	85 ± 0.7 a
Nicotiana tabacum	220 ± 93.5 a	59 ± 18.6 b	11 ± 3.8 b	70 ± 33.9 a
Tephrosia vogelii	218 ± 50.6 a	96 ± 0.5 a	78 ± 13.9 a	1 ± 0.4 b

Results

Effect on *Callosobruchus maculatus* beetles

In the oviposition bioassay, all plant powders reduced the number of *C. maculatus* eggs laid on treated beans (Table 2). When applied after oviposition, *Nicotiana tabacum* had an ovicidal effect, which caused the percentages of hatched eggs and emerging adults to be lower than from untreated beans. *Tephrosia vogelii* did not have any effect on the developing stages of the beetle, but once the adults emerged and were exposed to the plant powder, they died within a short period.

Table 3: A) Effects of plant powders and oils on parasitisation rate and adult longevity (beans treated before release of the parasitoids) and B) effect on the percentage of offspring emerging for *Uscana lariophaga* egg parasitoids (beans treated after parasitisation) (means of five or six replications ± S.D.). Values in columns followed by the same letter are not different ($\alpha = 0.05$).

A	Powders	Number of eggs parasitised per wasp	Number of dead wasps out of 10 after 24 h
	Control	6.67 ± 4.89 a	2.7 ± 2.16 ab
	Azadirachta indica	3.22 ± 3.07 b	3.9 ± 1.83 abc
	Blumea aurita	0.57 ± 0.88 c	5.5 ± 1.87 bc
	Nicotiana tabacum	0.00 ± 0.00 c	10.0 ± 0.00 d
	Tephrosia vogelii	0.00 ± 0.00 c	10.0 ± 0.00 d
	Oils		
	Control + ethanol	7.3 ± 7.29 a	
	Azadirachta indica oil	0.4 ± 0.74 b	
	Clausena anisata	5.6 ± 5.34 a	
	Cymbopogon MIX	0.0 ± 0.00 b	
	Cymbopogon nardus	0.0 ± 0.00 b	
	Cymbopogon schoenanthus	4.0 ± 6.02 ab	
	Ocimum basilicum	5.4 ± 6.84 a	

B	Powders	Emergence from treated parasitised eggs (%)
	Control	84 ± 12.4 a
	Azadirachta indica	82 ± 17.1 a
	Blumea aurita	82 ± 10.2 a
	Nicotiana tabacum	0 ± 0.0 b
	Tephrosia vogelii	47 ± 15.5 c

Effects on *Uscana lariophaga* egg parasitoids

In the test for inhibitory effects on oviposition, the number of eggs parasitised by *U. lariophaga* was reduced by all powder treatments (Table 3A). The powders of *Nicotiana tabacum* and *Tephrosia vogelii* completely prevented parasitisation. The oils of *Cymbopogon* MIX, *Cymbopogon nardus* and to a lesser extent *Azadirachta indica* prevented parasitisation when compared to alcohol treated control beans. Complete adult mortality was observed within 24 h after the introduction of the parasitoids on beans treated with powders of *Nicotiana tabacum* and *Tephrosia vogelii*. Adult mortality was high on *Blumea aurita* treated beans as well. If the beans were treated after parasitisation, the powder of *Tephrosia vogelii* partly and *Nicotiana tabacum* completely prevented emergence of the parasitoids (Table 3B).

The results of the repellence test are shown in Figure 1. The oils of *Azadirachta indica*, *Cymbopogon* MIX, *Cymbopogon nardus*, *Ocimum basilicum* and to a lesser extent *Clausena anisata* were repellent to the wasps.

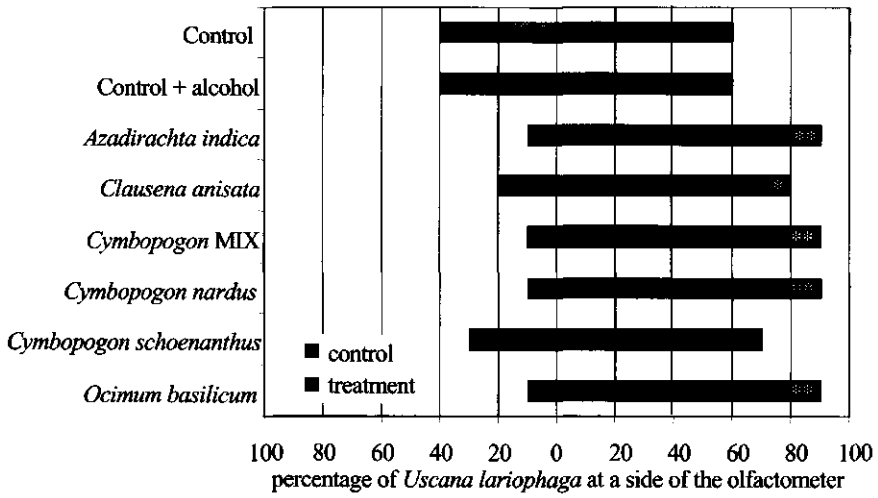

Figure 1: Results of the repellence test with Uscana lariophaga in an I-tube olfactometer. Each bar represents a total of 10 replicates (*: $p < 0.05$, **: $p<0.01$)

Effects on *Dinarmus basalis* larval parasitoids

In the test for inhibitory effects on oviposition, the powders of *Nicotiana tabacum* and *Tephrosia vogelii* strongly reduced parasitisation of beetle larvae by *D. basalis* and caused mortality of adult parasitoids within 24 hours (Table 4). However, if the beans were treated

after parasitisation, the percentage of emerging wasps was equal for all treatments and the control.

In the olfactometer test with *D. basalis*, no effect was found for any of the treatments (Figure 2).

Table 4: A) Effects of plant powders and oils on parasitisation and adult longevity (beans treated before release of the parasitoids) and B) effect on the percentage of offspring emerging for *Dinarmus basalis* (beans treated soon after parasitisation) (means of six replications ± S.D.). Values in columns followed by the same letter are not different (α = 0.05).

A	Plant powder	Number of larvae parasitised	Adult mortality (%)	
			1 DAT[a]	3 DAT
	Control	59 ± 25.8 a	28 ± 27.8 a	69 ± 28.7 a
	Azadirachta indica	60 ± 16.0 a	42 ± 25.3 a	69 ± 34.0 a
	Blumea aurita	64 ± 23.5 a	36 ± 24.5 a	72 ± 17.2 a
	Nicotiana tabacum	0.2 ± 0.41 b	100 ± 0.0 b	100 ± 0.0 a
	Tephrosia vogelii	0.2 ± 0.41 b	97 ± 6.8 b	100 ± 0.0 a

B	Plant powder	Emergence from treated parasitised beans (%)
	Control	51 ± 27.2 a
	Azadirachta indica	44 ± 29.2 a
	Blumea aurita	55 ± 30.2 a
	Nicotiana tabacum	39 ± 3.5 a
	Tephrosia vogelii	40 ± 6.6 a

a: DAT = Days after treatment

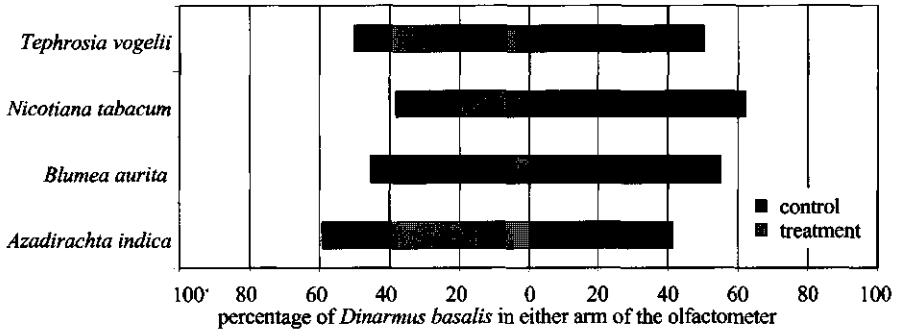

Figure 2: Repellence test with Dinarmus basalis wasps. The percentage of wasps going to either arm of the Y-tube olfactometer as influenced by hosts in beans treated with plant powders.

Study on seeds collected from granaries

The beans that had been stored without any treatment before the sampling, were infested by bruchid beetles and their parasitoids before the treatments were applied (Table 5). The number of bruchid eggs was comparable for the first and the eighth day after treatment, but it was higher at 24 days after treatment for all treatments except for *Tephrosia vogelii* where the number of eggs remained did not increase at all.

The percentage of eggs that was parasitised was between 2 and 3 percent at the time of treatment. This percentage had decreased after 8 and 24 days for beans treated with *Azadirachta indica*. After eight days, the percentage of egg parasitisation was lower than in the control for beans treated with *Azadirachta indica* or *Nicotiana tabacum*.

The number of bruchid beetles decreased in the first week and increased after that time for all samples. The most dramatic increase was measured in beans treated with *Tephrosia vogelii*. The number of adult *D. basalis* parasitoids increased with time for all treatments except for the beans treated with *Tephrosia vogelii*.

Table 5: Numbers of eggs and adults for *Callosobruchus maculatus* and larval parasitoids and the percentage of eggs parasitised on one hundred seeds of cowpea and their changes in time after treatment with plant powders. Small letters behind the values refer to effects of time within a treatment; capital letters refer to differences between treatments within the same time point. Values are means of six replications ± SD. Values in columns followed by the same letter are not different ($\alpha = 0.05$).

Treatment	DAT[a]	Bruchid eggs	Eggs parasitised (%)	Bruchid adults	*Dinarmus* adults
Control	0	206 ± 22.1 aA	2.8 ± 2.99 aA	65 ± 66.1 abA	9.0 ± 4.90 aA
	8	244 ± 49.4 aA	4.0 ± 2.76 aA	14 ± 7.3 aA	14.5 ± 3.15 aA
	24	465 ± 201.2 bA	0.9 ± 0.54 aA	90 ± 61.0 bAB	80.3 ± 51.25 bA
Azadirachta indica	0	258 ± 53.1 aA	2.3 ± 1.05 aA	48 ± 48.6 abA	11.7 ± 13.50 aA
	8	194 ± 42.6 aA	1.1 ± 0.64 bB	14 ± 8.4 aA	14.0 ± 3.03 aA
	24	334 ± 45.6 bAB	0.6 ± 0.23 bA	69 ± 20.3 bAB	69.3 ± 8.96 bA
Blumea aurita	0	214 ± 46.8 aA	2.9 ± 3.46 aA	58 ± 75.5 abA	10.7 ± 12.31 aA
	8	207 ± 36.6 aA	1.6 ± 1.22 aAB	14 ± 5.0 aA	14.2 ± 4.02 aAB
	24	401 ± 101.0 bAB	1.0 ± 0.89 aA	57 ± 21.6 bA	62.3 ± 18.12 bA
Nicotiana tabacum	0	203 ± 58.9 aA	2.2 ± 1.73 aA	38 ± 28.2 aA	9.5 ± 5.17 aA
	8	217 ± 42.6 aA	0.8 ± 0.59 aB	13 ± 3.0 aA	11.7 ± 2.25 aAB
	24	399 ± 164.7 bAB	0.3 ± 0.36 aA	129 ± 27.3 bBC	15.7 ± 5.68 aB
Tephrosia vogelii	0	215 ± 40.4 aA	2.3 ± 1.37 aA	39 ± 40.9 aA	10.5 ± 7.20 aA
	8	203 ± 25.6 aA	2.8 ± 2.15 aAB	10 ± 5.3 bA	9.0 ± 2.83 aB
	24	192 ± 31.6 aB	0.5 ± 0.42 aA	164 ± 23.7 cC	8.0 ± 3.58 aB

a: DAT= days after treatment

Discussion

The plants tested in this study are all traditionally used as protectants against insect damage and they were selected based on their efficacy against the beetle *C. maculatus* (Chapter 3 and 4). From the present study, it appears that these plant products all had negative effects on the beetles' parasitoids as well. These effects came about either directly as a contact poison to the wasps or more indirectly through effects on the developmental success of the parasitoids.

For the powders, contrary to the results presented here, earlier research on different samples of the plant species revealed that *Azadirachta indica* and *Blumea aurita* did not have any influence on the number of eggs laid by the beetle. For *Nicotiana tabacum* and *Tephrosia vogelii*, the results obtained here were similar to earlier findings. For *Nicotiana tabacum*, the complete lack of offspring was due only to an ovicidal effect, whereas for *Tephrosia vogelii*, the adult beetles died soon after they contacted the powder and thus laid only few eggs (Chapter 3). Similar to the effect on the beetles, adults of the parasitoids *U. lariophaga* and *D. basalis* were most affected by powders of *Tephrosia vogelii* and *Nicotiana tabacum*, whereas the other two powders had weaker effects. *Blumea aurita* was harmful only to *U. lariophaga*.

All oils had a negative effect on the number of eggs laid by *U. lariophaga*, whereas in a no-choice situation the beetles' oviposition was only impaired by *Cymbopogon* MIX, *Cymbopogon nardus* and *Cymbopogon schoenanthus* (Chapter 4). The negative effect on the parasitoids' oviposition is at least partly explained by the fact that the bruchid eggs were killed by the treatment with 70% ethanol. However, the numbers of eggs that were parasitised were lower for the oil treatments than for the ethanol treated control, so the oils themselves, especially *Azadirachta indica* and the *Cymbopogon* oils had an oviposition inhibiting effect. In the two-choice repellence test, the ethanol treatment did not affect the orientation of *U. lariophaga* whereas the wasps were repelled by all oils except *Cymbopogon schoenanthus*. Different from *U. lariophaga*, the beetles were not repelled by *Azadirachta indica* (Chapter 4).

The olfactometer tests with *D. basalis* did not show any effect of plant powders on the wasps' behaviour. This lack of results might have been caused by the set-up we used. The Y-tube olfactometer has been used successfully to determine odour preferences of mites (Takabayashi and Dicke, 1992) and of parasitoids (Bin *et al.*, 1987). Wäckers and Swaan (1993) reported the successful use of this olfactometer for the parasitoid wasp *Cotesia rubecula*, but the same set-up was not suitable to investigate the response of *Cotesia glomerata* to infochemicals (Steinberg *et al.*, 1992). The use of an Y-tube olfactometer in tests with *D. basalis* has not previously been reported.

In the study on seeds collected from granaries, the adults of *U. lariophaga*, if they were present, could not be counted reliably. Due to their small size, they were difficult to observe among the infested beans and they might have escaped from the cloth-covered jars. The

presence of egg parasitoids could more easily be established on the beans where the parasitised eggs turn black whereas unparasitised eggs are white or colourless. The parasitism by the bigger larval parasitoid *D. basalis* could only be estimated from the number of adult parasitoids emerging. The mortality of developmental stages of the wasp could not be determined, since the development takes place inside the beetle larva in the bean.

The effect of powder of *Tephrosia vogelii* on adult bruchid beetles seemed to be less pronounced in this longer-term study on seeds collected from granaries than in the other experiments we conducted. An explanation might be that in the relatively open environment of a cloth-covered glass jar the insecticidal components evaporate. We hypothesise that the first generation of adult insects on the bean surface was killed including the parasitoids. No new eggs were laid, but the ones that had been laid already, were unaffected. Parasitoids would then not survive after the first generation, since there are no host eggs or larvae. The beetles, with a longer life-cycle emerge after the insecticide has lost its effect.

The parasitoids we used are not the only ones that parasitise *C. maculatus*, but they are important representatives of the two largest families of specialist parasitoids of bruchids (Van Huis, 1991). *Uscana lariophaga* is the most abundant egg parasitoid in West Africa (Van Alebeek, 1996) whereas *D. basalis* is an abundant larval parasitoid that is more effective in controlling the beetle population than other larval parasitoids such as *Eupelmus vuiletii* (Sanon and Ouedraogo, 1998).

Southgate (1978) reported sceptically on the use of parasitoids in the protection of stored seeds since these wasps would need a long standing population of fairly high numbers of the pest before they could become effective and if these conditions would be fulfilled, the damage would be unacceptable anyway. However, the percentage of parasitism by *U. lariophaga* in the field was 27% (Alzouma, 1989) to 67%, which made this species more important as a cause of mortality than larval parasitoids (Sagnia, 1994). In storage, this parasitoid was able to keep the beetle population at a low number, with more than 80% of the eggs being parasitised (Van Huis *et al.*, 1998). When *D. basalis* parasitoids, that were present at harvest in small numbers, were deliberately introduced into the storage room in larger numbers, they effectively reduced the damage done by bruchid beetles (Sanon *et al.*, 1998, 1999). The intrinsic rate of growth of a *D. basalis* population was always higher than that of its host, independent of the variations in climatic conditions during the dry season (Sanon *et al.*, 1998).

Under ambient conditions in Niger, adults of *U. lariophaga* lived for up to 6 days (Van Huis *et al.*, 1998), but the longevity was dependent on the temperature. At 30 °C, the mean longevity was 1.5 days, whereas at 25 °C these wasps could reach 2.7 days of age and at 12.5°C even 13.2 days (Van Huis *et al.*, 1994). The longevity of *D. basalis* adults, when deprived of food and water as in our experiment was 15.5 days on average and when

supplied with possible hosts, the wasps lived on average more than 23 days (Schmale *et al.*, 2001). The early death of the parasitoids in our experiments could be due to the frequent disturbance for the observations, but it is also at least partly caused by the treatment applied to the beans.

The method of storing seeds with insecticidal plants is not in all cases compatible with biological control strategies. Especially *U. lariophaga* seemed sensitive to the effects of the botanicals whereas *D. basalis* was not repelled by the treatments. The powders of *Nicotiana tabacum* and *Tephrosia vogelii* were effective against the beetle host, but they also effectively prevented parasitisation. The release of parasitoids in the storage room could better be combined with powders of *Azadirachta indica* or *Blumea aurita* since these powders had a more pronounced effect on the beetle than on its parasitoids. Especially the latter botanical could be promising, since it showed a strong repellent effect on beetles but the effect on *U. lariophaga* and *D. basalis* is absent or only weak, depending on the parameter evaluated.

Acknowledgements

The research was financed by the Centre Béninois pour le Développement Durable. We thank L. Saka and H. Kassa for their assistance in the laboratory. The critical comments on the manuscript by M. Dicke and A. Van Huis were highly appreciated.

Chapter 6

Safety evaluation of neem (*Azadirachta indica*) derived pesticides

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Abstract

The neem tree, *Azadirachta indica* provides many useful products that are used as pesticides and could be applied to protect stored seeds against insects. In this study we present a review of the toxicological data from human and animal studies with mostly oral or per os administration of different neem-based preparations. Beneficial effects, such as blood sugar lowering properties, anti-parasitic, anti-inflammatory, anti-ulcer and hepatoprotective effects are often reported, but toxic effects were also found for all preparations. For all preparations, reversible effects on reproduction of both male and female mammals are reported. From the available data, safety assessments for the various neem-derived preparations were made and the outcomes are compared to the ingestion of residues on food treated with neem preparations as insecticides. This leads to the conclusion that, if applied with care, use of neem as an insecticide should not be discouraged.

Key words: Toxicity, health evaluation, oral administration, NOAEL, neem-derived pesticides

1 Introduction

In the search for environmentally safe pesticides, during the last decades, 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 as a protective agent for a crop against insects is often traditional and age-old.

The one plant species that is best investigated for its effects against insects is probably the neem tree, *Azadirachta indica* Juss. (Meliaceae). All parts of this tropical tree contain bitter compounds (Chawla *et al.*, 1995; Van der Nat *et al.*, 1991) that often have an antifeedant effect and can interfere with hormonal processes in insects (Ascher, 1993; Schmutterer,

1990). Crude extracts or unprocessed parts of this plant are mixed with seeds such as maize, grain, rice and beans in storage for protection against seed-eating insects. Results of storage tests mostly indicate that the leaf powder, the seed oil and all kinds of extracts do indeed have a negative effect on the development and survival of insects.

However, if neem products are to be used to treat stored seeds against insects, the mammalian consumers of these seeds ought not to be affected by residues of this treatment. Much controversy exists about the use of especially the seed oil of the neem tree. In some reports, it is claimed that the oil is easily removed from the seeds and that it does not affect the palatability of the beans (Anonymous, 1995). In other reports however, it is mentioned that it is hardly possible to remove the very bitter oil from treated seeds, and that germination of treated seeds is negatively influenced (Naik and Dumbre, 1985). Moreover, the oil can turn rancid (De Groot, 1991) and may be contaminated with aflatoxins (Sinniah *et al.*, 1982) derived from seed-infesting fungi.

Given the use of various neem-derived products as pesticides and the realistic chance that residues derived from the treatments will still be present at the time of consumption, it would be of interest to obtain insight in the possible resulting consumer risks. Therefore, in this study we present a review of the toxicological data from human and animal studies with oral or per os administration of different neem-based preparations. These preparations can consist of unprocessed plant parts, the seed oil, aqueous extracts of parts of the tree, extracts obtained with non-aqueous solvents, the pure bioactive insecticide ingredients and commercially available neem-based pesticides.

The first three application modes would be best applicable for low-resource farmers in tropical countries, where no complex extractions can be performed due to a lack of appropriate solvents and equipment. The other application modes could be valuable in countries where law requires exact definition of the ingredients of pesticides. Preparations of seed cake, the rest-product after oil extraction, are left out here, since most active insecticidal principles are removed from this material and the effects on mammals are usually little pronounced (Gangopadhyay *et al.*, 1979; Nath *et al.*, 1989; Ramu *et al.*, 1997).

A list of abbreviations is given at the end of this paper.

2 Unprocessed material

Many parts of the neem tree are used in unprocessed form. For such application, raw plant materials, mainly leaves and fruit kernels are harvested and applied immediately or after drying or grinding. The toxic components in these materials are not concentrated and the toxicity is expected to be less pronounced than in extracts. In areas where the tree grows, accidental ingestion of these materials by grazing cattle or playing children is more likely than for processed material, since the tree is wide spread and rather common.

2.1 Effects on humans

Table 1 summarises the reported effects of unprocessed neem materials on humans. Alam *et al.* (1990) reported that the use of neem leaves against diabetes in indigenous medicine in India is highly satisfactory to the local population. A disadvantage of such self-medication is reported by Kadiri *et al.* (1999) who found that traditional neem leaf-based medicines, taken to treat febrile illness, abdominal upset or to induce abortion or infertility had acute toxic effects. The major features observed were oliguria or anuria, jaundice and anaemia. The picture was consistent with acute tubular necrosis in all the cases and the mechanisms causing the effects were intravascular haemolysis, hepatotoxicity and direct nephrotoxicity. Three out of 53 patients died. Another disadvantageous feature was found in the allergenicity of the pollen. Chakraborty *et al.* (1998), in a survey of the aeropalynoflora in India, identified 46 pollen types. The abundance of pollen of the neem tree was relatively low, but when subjected to clinical investigation to determine their degree of allergenicity on adult respiratory allergic patients, they appeared to be highly allergenic.
Since none of these reports mentioned any quantitative exposure data, they cannot be used to assess the risks associated with the exposure to unprocessed neem materials.

Table 1: Effects of unprocessed neem materials on humans

Plant part	Administration	Dose	Duration	Observed effect(s)	Reference
Leaves	Oral	2 tablets	*	Anti-diabetic effect	Alam *et al.*, 1990
Pollen	Skin prick test	*	Once	Allergenic effect	Chakraborty *et al.*, 1998
Leaves	Oral, intravaginally	*	*	Acute renal failure	Kadiri *et al.*, 1999

* = not specified in reference

2.2 Effects on animals

The effects of unprocessed neem materials on animals are summarised in Table 2. Acute toxicity of unprocessed material in animals was reported only for a sheep that ate neem leaves. Ingestion resulted in nervous symptoms (head movements, walking in circles) with dyspnoea, an increase in body temperature, hepatic failure and tympanites. The symptoms lasted for 12 hours and were followed by the death of the animal (Ali and Salih, 1982).
More positively, administration of leaf sap caused an anti-anxiety effect in rats at low doses, while high doses did not cause such effect (Jaiswal *et al.*, 1994). In sheep, kernel powder caused a decrease in the number of nematode eggs in their faeces and an increase in body weight (Ahmed *et al.*, 1994).

Table 2: Effects of unprocessed neem materials on animals

Plant part	Test animal	Dose	Duration (days)	Observed effect(s)	Reference
ACUTE EFFECTS					
Kernel powder	Sheep	75; 100 mg/kg bw	1	Effect against intestinal nematodes	Ahmed *et al.*, 1994
Leaves	Sheep	100 g/sheep	1	Acute toxicity	Ali and Salih, 1982
Leaf sap	Rats	10 - 800 mg/kg bw	1	Anti-anxiety effect	Jaiswal *et al.*, 1994
SUBACUTE EFFECTS					
Leaf powder	Rats	100 mg/rat	24	Changes in testes	Joshi *et al.*, 1996
Leaf powder	Rats	20; 40; 60 mg/rat	24	Reduced epididymal function	Kasturi *et al.*, 1995
Leaf powder	Rats	20; 40; 60 mg/rat	24	Reduced weight seminal vesicles and ventral prostate	Kasturi *et al.*, 1997
Leaf powder	Rats	20; 40; 60 mg/rat	24	Reduced sperm count and motility, sperm malformations	Parveen *et al.*, 1993
CHRONIC EFFECTS					
Leaf powder	Rats	100 mg/rat	48	Sperm parameters	Aladakatti *et al.*, 2001
Leaves	Cattle	10; 20; 30 % in diet	98	Effect against intestinal nematodes	Pietrosemoli *et al.*, 1999
Fruits	Rabbits	2000 mg/rabbit/d	70	Control hydatidosis	Tanveer *et al.*, 1998

Neem leaf powder had an important effect on the reproductive ability of male rats. Leaf powder caused a decrease in the weight of the seminal vesicle and the ventral prostate (Kasturi *et al.* 1997), a reduction in the sperm count and sperm motility as well as an increased percentage of malformed sperm (Parveen *et al.*, 1993). Moreover, at a dose of 100 mg/rat, the diameters of the seminiferous tubule and nuclei of germinal elements reduced, mass atrophy of spermatogenic elements were observed and the Leydig cells atrophied. Gradual recovery in histological and biochemical parameters was found after termination of the treatment (Joshi *et al.*, 1996). At slightly lower dosages, the height of the epithelium and the diameter of the nucleus in caput and cauda epididymis were reduced dose-dependently. The lumen of the caput was packed with lymphocytes and the serum testosterone concentration was decreased (Kasturi *et al.*, 1995). Biochemically the powder caused

decreases in protein content and acid phosphatase activity, and increases in activities of alkaline phosphatase and lactate dehydrogenase (Kasturi *et al.*, 1997) and the contents of total free sugar, glycogen and cholesterol (Joshi *et al.*, 1996).

In a chronic study, Aladakatti *et al.* (2001) found that leaf powder in rats caused a decrease in total sperm-count and in motility and forward velocity of sperm cells. The relative percentage of abnormal sperm increased and the fructose content of caudal semen of the epididymis decreased. Since the effects of the powder were annihilated when testosterone was administered simultaneously, the authors suggested that the effects were due to an androgen deficiency, caused by the anti-androgenic property of the neem leaves, thereby affecting the physiological maturation of sperm.
A positive effect against intestinal nematodes was found for cattle upon neem leaf feeding without any effect on weight gain (Pietrosemoli *et al.*, 1999). In rabbits, neem fruits caused decreased serum activities of acid phosphatase, alkaline phosphatase and glucose and an improvement of SGOT, SGPT, cholesterol, total protein and bilirubin values (Tanveer *et al.*, 1998).

2.3 Conclusion

All together, the major animal-study-derived toxic outcome of unprocessed neem materials may be the effects on male fertility upon sub-acute or semi-chronic exposure to the leaf powder at 20 to 100 mg/rat. In the multiple-dose studies, rats weighing 230 -250 g were administered 20, 40 or 60 mg each.
The relative weight of the testis, the epididymis (Parveen *et al.*, 1993), and the caput and cauda of the latter (Kasturi *et al.*, 1995) were not affected at any dose. For these parameters, the NOAEL would thus be 60 mg/rat which for rats of 250 gram amounts to 240 mg/kg bw. The total number of sperm cells/ml (Parveen *et al.*, 1993) and the protein content of the caput of the epididymis (Kasturi *et al.*, 1995) were affected only at doses of 20 and 40 mg unprocessed leaves/rat respectively (= 80 and 160 mg/kg bw). The other parameters measured were much affected even at the lowest dose. The NAELs calculated using loglinear extrapolation ranged from $2*10^{-14}$ mg/kg bw for the LDH concentration in the seminal vesicle (Kasturi *et al.*, 1997) to 355 mg/kg bw for the motile sperm count (Parveen *et al.*, 1993). The LOAEL from all these studies is 20 mg/rat or rather 80 mg/kg bw. If a factor 10 is used for extrapolation of the LOAEL to a NAEL this results in a value of 8 mg/kg bw. Extrapolating this to the human situation using safety factors for intra- and interspecies extrapolations this results in an acceptable daily intake of 0.1 * 0.1 * 8 mg/kg amounting to 0.08 mg/kg bw. For a 70 kg weighing human adult this amounts to 70 x 0.08 mg/kg =5.6 mg unprocessed leaves/day. This value gives an indication of the range in which a safe dose for daily human exposure could be found.

3 Oil

The seeds of the neem tree are rich in oil, which can be extracted by hand or mechanically. In many tropical countries, this oil is sold as a household medicament for use against all kinds of inconveniences and diseases such as muscle-aches, malaria, tuberculosis and even diabetes.

3.1 Effects on humans

In Table 3 the reported effects of neem oil on humans are summarised. Two cases were described where oral administration to young children resulted in acute toxic effects. The oil, even in small amounts was reported to cause toxic encephalopathy. Features were vomiting, drowsiness, tachypnoea, and recurrent generalised seizures. Laboratory tests showed that the oil causes leukocytosis and metabolic acidosis (Lai *et al.*, 1990). Sinniah *et al.* (1982) reported the case of a child, who died after administration of the oil as treatment for a cough. Autopsy findings revealed changes in the liver and kidneys consistent with Reye's syndrome but unlike those described in acute aflatoxicosis. As aflatoxins have been identified in oil samples, the toxic action of the oil may have been due to the synergistic effects of aflatoxins and other toxic components in the oil.

In tribal medicine in India, the oil is considered to have a contraceptive activity (Lakshmanan and Naryanan, 1990). Other studies in India revealed that the oil is effective as a mosquito-repelling agent, and thus helps to prevent malaria. The oil, either applied topically (Kant and Bhatt, 1994; Mishra *et al.*, 1995; Sharma *et al.*, 1993) or burnt in a lamp (Sharma and Ansari, 1994) provided protection from *Anopheles* and *Culex* mosquito species. The doses reported in the table (5 and 12 ml per young child) are clearly toxic, which implies that threshold values for human consumption calculated from animal studies should be at least much below ± 0.20 ml/kg bw (taking 25 kg for the bw of a child).

Table 3: Effects of neem oil on humans

Administration	Dose	Duration	Observed effect(s)	Reference
Oral	'Droplets' and 5 ml	Once	Encephalopatic effect	Lai *et al.*, 1990
Oral	12 ml	2 days	Acute toxicity	Sinniah *et al.*, 1982

3.2 Effects on animals

The effects of oral administration of oil to animals are summarised in Table 4. Gandhi *et al.* (1988) reported acute toxicity after ingestion of the oil by rats and rabbits. The oil induced dose- and time-dependent effects on motor activity, respiration and on orientation within the cage and the animals had diarrhoea, tremors and convulsions. At doses above 5 ml/kg bw death occurred within 24 hours. The LD_{50} value was 14.1 ml/kg bw for rats and, showing

similar symptoms, 24.0 ml/kg bw for rabbits. The serum levels of bilirubin and SGOT in treated rats were elevated. The stomach retained most of the oil and the vital organs were unaffected, except for the lungs where a collapse of alveoli was seen along with a thickening of inter-alveolar septa and congestion and haemorrhage into the air spaces. The oil was not toxic to mice at lower doses, but at high dose treated animals showed hyper-excitability to sound and touch, convulsive jerks, laboured respiration and some animals died (Tandan *et al.*, 1995).

In rats, administration of neem oil during the first few days of pregnancy had a stronger abortive effect than later administration. At a dose of 6 ml/kg, 3 out of 13 adult animals died (Lal *et al.*, 1987). Administration of oil increased tail flick reaction time and reduced induced writhing (Khosla *et al.*, 2000a). In normal and hyperglycaemic rats, administration of oil caused a lowering of the blood glucose level (Dixit *et al.*, 1986).

Table 4: Effects of neem oil on animals

Test animal	**Dose**	**Duration (days)**	**Observed effect(s)**	**Reference**
ACUTE EFFECTS				
Rats	200 mg/rat	1	Anti-diabetic effect	Dixit *et al.*, 1986
Rabbits, rats	5 - 80 ml/kg bw	1	Acute toxicity	Gandhi *et al.*, 1988
Rats	2 ml/kg bw	1	Anti-nociceptive effect	Khosla *et al.*, 2000a
Rats	4; 6 ml/kg bw	2 - 3	Abortive effect	Lal *et al.*, 1987
Mice	1.0 - 28.2 g/kg bw	1	Toxicity	Tandan *et al.*, 1995
SUBACUTE EFFECTS				
Rats	2.0; 3.3; 4.6 ml/kg bw	18	Anti-fertility in females	Dhaliwal *et al.*, 1998
Rabbits	5 ml/kg bw	28	Anti-diabetic effect	Khosla *et al.*, 2000b
CHRONIC EFFECTS				
Rats	10 % in diet	3 generations, 130 each	No toxicity, no effect on reproduction	Chinnasamy *et al.*, 1993

A subacute effect was that the oestrous cycle of female rats was disturbed resulting in a reduction in fertility. The body weight was reduced when the animals were administered a high dose of neem oil (Dhaliwal *et al.*, 1998). Subchronic administration of seed oil led to lowered blood sugar levels in normal and diabetic rabbits (Khosla *et al.*, 2000b).

Chronic effects are described by Lakshminarayana (1987) who investigated the neem tree as a potential source of oil and by Rukmini (1987) who concluded from a study in rats that the

neem tree would provide a safe source of edible oil. Debitterised neem oil was found useful as animal feed (Rukmini *et al.*, 1991). A study over three generations with male and female rats fed a diet containing debitterised oil did not show any adverse effects on the general health or reproductive parameters. The mean organ weights and the histopathological evaluation of all the organs were similar. The extract was negative in the Ames test for carcinogenicity (Chinnasamy *et al.*, 1993).

3.3 Conclusion

Neem oil shows acute toxicity at relatively high doses of 14 and 24 ml/kg bw for rats and rabbits respectively. Upon use as an insecticide, it is unlikely that these high levels of intake will be encountered when considering human intake of residues on treated beans, since these doses in rats and rabbits would amount to about 1000 ml of oil or more for a 70 kg human adult. The most relevant adverse effect reported in neem-oil exposed animals seems to be the anti-fertility effect in female rats observed upon sub-chronic exposure to 2.0 - 4.6 ml/kg bw. At 2.0 ml/kg bw the oestrous cycle of rats was disturbed, but no other effects were found (Dhaliwal *et al.*, 1998). This implies a LOAEL for the anti-fertility effect in female rats of 2.0 ml/kg bw. Taking a factor of 10 for extrapolation of the LOAEL to a NAEL this results in a value of 0.2 ml/kg bw. Extrapolating this to the human situation using safety factors for intra- and interspecies extrapolations this results in an acceptable daily intake of 0.1 * 0.1 * 0.2 ml/kg amounting to 0.002 ml/kg bw. This implies that a daily intake of 0.14 ml oil for an adult of 70 kg can be considered safe. This value of 0.002 ml/kg is 100 times lower than the dose of 0.2 ml/kg reported to be toxic in children.

4 Aqueous extracts

A simple way to prepare a plant extract is the soaking of plant material in water. This provides aqueous neem extracts.

4.1. Effects on humans

Kroes *et al.* (1993) reported that in Sri Lankan medicine a fermented decoction of neem bark is taken as a drug with immunomodulatory activity. An in vitro haemolytic assay proved that the human complement system (the classical and the alternative pathways) and the chemiluminescence from zymosan-activated polymorphonuclear leukocytes from healthy volunteers were inhibited.

4.2 Effects on animals

As shown in Table 5, the investigations on effects of aqueous neem extracts on animals are numerous, and in most cases beneficial instead of harmful. Leaf extract caused a moderate decrease of the blood glucose levels in mice (Mossa, 1985). It produced hypoglycaemia in

normal rats, while in diabetic rats there was a decrease in blood sugar. However, the extract had toxic effects, as reflected in body weight loss and high percentage mortality. The clotting time of blood was longer than normal. Serum cholesterol level increased with a concomitant decrease in liver fat and a dose-related drop in liver proteins (El Hawary and Kholief, 1990).

Leaf extract was effective against *Plasmodium yoelii nigeriensis* in mice (Obaseki and Jegede Fadunsin, 1986). However, Abatan and Makinde (1986) found at best a slight suppression of *Plasmodium berghei* after application of leaf extract to mice but they did not find any prophylactic effect.

The tail flick reaction time increased and a reduction in induced writhing was observed in rats that were administered leaf extract. Naloxone pre-treatment partially reversed the effects. The effects of leaf extract were more pronounced than those of seed oil (Khosla *et al.*, 2000a). Leaf extract reduced gastric ulcer severity in rats and decreased gastric mucosal damage (Garg *et al.*, 1993a). It stimulated GGT activity and nearly normalised stress-induced suppression of GGT in the lymphoid system (Koner *et al.*, 1997). Induced carcinogenesis with accompanying high levels of lipid peroxidation and low levels of GSH, GPx, GST and GGT in rats could be effectively treated with leaf extract. A five-day pre-treatment with leaf extract decreased the formation of lipid peroxides and enhanced the levels of antioxidants and detoxifying enzymes in the stomach, the liver and circulation (Arivazhagan *et al.*, 2000a). Upon longer administration, such leaf extract had similar effects (Arivazhagan *et al.*, 1999a, 1999b, 2000b).

Table 5: Effects of aqueous neem extracts on animals

Plant part	**Test animal**	**Dose (equivalent weight (mg) of leaves; fresh (f) or dry (d))**	**Duration (days)**	**Observed effect(s)**	**Reference**
ACUTE EFFECTS					
Leaves	Mice	125 - 500 mg/kg bw (166 - 666.7; f)	4	Slight effect against malaria parasites	Abatan and Makinde, 1986
Leaves	Rats	250 mg/kg bw (250; f)	5	Reduced lipid peroxidation, increased antioxidant status	Arivazhagan *et al.*, 2000a
Leaves	Rats	200; 300 mg/kg bw (200; 300; d)	7	Anti-diabetic effect and toxicity	El Hawary and Kholief, 1990
Leaves	Rats	10 - 160 mg/kg bw (10 - 160; d)	1	Gastric anti-ulcer effect	Garg *et al.*, 1993a

Part part	Test	Dose	Duration	Observed effect(s)	Reference
Leaves	Rats	100 mg/kg bw (*; *)	5	Stimulation of GGT activity	Koner *et al.*, 1997
Leaves	Mice	0.5 ml/mouse (12.5 mg; d)	1	Anti-diabetic effect	Mossa, 1985
Leaves	Mice	100 - 400 mg/kg bw (690 -2778; d)	1	Effective against malaria parasites	Obaseki and Jegede Fadunsin, 1986
SUBACUTE EFFECTS					
Leaves	Rats	500 mg/kg bw (*; d)	9	Hepatoprotective effect	Bhanwra *et al.*, 2000
Leaves	Rabbits	500 mg/kg bw (*; d)	28	Anti-diabetic effect	Khosla *et al.*, 2000b
Leaves	Mice	40; 100 mg/kg bw (200; 500; d)	20	Adverse effect on thyroid function	Panda and Kar, 2000
Leaves	Mice	10; 30; 100 mg/kg bw (*; *)	21	Modulation of immune responses	Ray *et al.*, 1996
CHRONIC EFFECTS					
Leaves suspension	Goats, guinea pigs	50 - 2000 mg/kg bw (50 - 2000; f & d)	5; 56	Toxicity	Ali, 1987
Leaves	Rats	100 mg/kg bw (100; f)	182	Chemopreventive potential	Arivazhagan *et al.*, 1999a
Leaves	Rats	100 mg/kg bw (100; f)	182	Effects on circulating lipid peroxides and antioxidants	Arivazhagan *et al.*, 1999b
Leaves	Rats	250 mg/kg bw (250; f)	182	Lipid peroxidation and antioxidant status	Arivazhagan *et al.*, 2000b
Leaves	Rats	1000 mg/kg bw (*; *)	42	Anti-diabetic effect	Bajaj and Srinivasan, 1999
Leaves	Hamsters	100 mg/kg bw (*; *)	42* in 98 days	Anti-carcinogenic effect	Balasenthil *et al.*, 1999a
Leaves	Hamsters	100 mg/kg bw (*; *)	42* in 98 days	Anti-carcinogenic effect	Balasenthil, *et al.*, 1999b
Leaves	Rats	100 mg/kg bw (*; *)	49	Toxicity	Hore *et al.*, 1999
Twigs, fruits	Rats	0.1 - 1.6 % in diet (0.25 - 4 g; d)	70	Effect on blood constituents	Parshad *et al.*, 1994
Kernels	Mice	*	30	Anti-tubercular effect	Usha and Saroja, 2001

* = not specified in reference

Dose-dependent sub-acute effects were observed after administration of aqueous leaf extract to mice. The extract reduced tri-iodothyronine (T3) and increased serum thyroxine (T4) concentrations. Hepatic lipid peroxidation increased and glucose-6-phosphatase activity decreased while the activities of superoxide dismutase and catalase were enhanced (Panda and Kar, 2000). Treatment of mice had no influence on liver, spleen, thymus or body weight indices but it caused elevated IgM and IgG concentrations and anti-ovalbumin antibody titres and it enhanced macrophage migration inhibition and footpad thickness (Ray *et al.*, 1996). Livers of paracetamol-induced rats were normal in appearance and histology after administration of leaf extract. The extract caused a reduction of paracetamol induced hepatotoxic effects. It reduced high serum levels of SGOT, SGPT and GGT (Bhanwra *et al.*, 2000). Subchronic administration of leaf extract caused a decrease in blood sugar levels in normal and diabetic rabbits. The extract was more effective than seed oil (Khosla *et al.*, 2000b).

Rats treated with leaf extract showed decreased appetite, body weight and pupillary reflex. They were found depressed and the TEC and the blood glucose level were reduced. Histopathological studies revealed congestion in the liver, kidneys, lungs and brain (Hore *et al.*, 1999). The body weight of goats and guinea pigs decreased due to administration of leaves to their drinking water. Both acute and chronic toxicity were evident through signs of weakness, loss of condition and depression. Decreases in heart, pulse and respiratory rates were observed and diarrhoea, tremors and ataxia occurred in some animals. TEC, PCV and Hb decreased slightly, whereas the activities of SGOT, sorbitol dehydrogenase and the concentrations of cholesterol, urea, creatine and potassium increased. Liver and kidneys were most affected (Ali, 1987). However, treatment of rats with leaf extract resulted in decreases in total testosterone, total bilirubin and K^+ in serum. There were increases in PCV, mean corpuscular Hb concentration, red blood cell, white blood cell and lymphocyte counts, but no cytotoxic effects were observed (Parshad *et al.*, 1994).
Leaf extract suppressed oral DMBA induced carcinogenesis in hamsters (Balasenthil *et al.*, 1999a). It prevented the changes induced by the carcinogen. Tumours developed in fewer animals and they were fewer and smaller (Balasenthil, *et al.*, 1999b).
The aqueous leaf extract caused a fall in blood glucose levels in diabetic rats (Bajaj and Srinivasan, 1999). Administration of kernel extract to mice protected their tissues from the damage caused by *Mycobacterium tuberculosis* (Usha and Saroja, 2001).

4.3 Conclusion

The effects of aqueous extracts are ambiguous. Many of the studies do not report dose-effect relations. Mostly positive effects are mentioned, even after administration of high doses, but toxic effects of 200 mg/kg resulting in death of treated goats were also observed (Ali, 1987). Hore *et al.* (1999) mentioned negative side effects after exposure of rats to 100 mg/kg. A

LOAEL of 40 mg/kg was found for effects on thyroid function in mice (Panda and Karr, 2000). Effects on reproduction were only indirectly mentioned as a decrease in testosterone upon administration of 0.1 % neem extract in the food of rats (Parshad *et al.*, 1994).
Since the beneficial effects are found at comparable and often even higher doses than these negative effects, we cannot deduce a reliable NAEL from these LOAEL values.

5 Non-aqueous extracts

If non-aqueous solvents are available, these can be used to extract more apolar, possibly more active constituents from neem material. These extracts, containing the active compounds and not the ineffective bulk of plant material, are often more active than the crude materials they were obtained from.

5.1 Effects on humans

In Table 6, the effects of several non-aqueous neem extracts on humans are summarised. Via ammonium precipitation, it was possible to isolate the allergenically active components from neem pollen. A skin prick test on human volunteers revealed several major allergens in neem pollen extract (Karmakar and Chatterjee, 1994).
A pessary including neem leaf extract did not show any toxicity and there were no side-effects whereas it was effective in prevention of pregnancy in most of the volunteers (Talwar *et al.*, 1997). Bombarde and Bombarde (1994) reported the traditional use of neem as a drug to treat diabetic patients.

Table 6: Effects of neem extracts made with non-aqueous solvents on humans

Plant part	Administration	Dose	Duration	Observed effect(s)	Reference
Pollen	Skin prick test	*	Once	Allergenic effect	Karmakar and Chatterjee, 1994
Leaf	Intravaginally	*	7 days	Prevention of pregnancy	Talwar *et al.*, 1997

* = not specified in reference

5.2 Effects on animals

Effects of orally administered non-aqueous extracts on animals are summarised in Table 7. Food consumption and body weights of three rodent pests decreased when they were fed on maize, adulterated with neem products. Methanol extracts were more repellent than the powders (Oguge *et al.*, 1997). Methanol extracts of bark and leaves had a pronounced anti-inflammatory and a good antipyretic effect in rats and rabbits. A test for acute oral toxicity

in mice showed an LD_{50} value of approximately 13 g/kg bw (Okpanyi and Ezeukwu, 1981). Methanol bark extract established an anti-thrombotic effect in mice (Olajide, 1999).

Symptoms of acute toxicity of acetone leaf extract were defined as a decrease in spontaneous activity, respiratory rate and body and limb tone in mice. Decreased responses to the environment, piloerection, a crouching gait, diuresis and a dose-dependent hypothermia were observed as well. No anti-microbial activity was found (Singh *et al.*, 1987). Two fractions of an acetone leaf extract showed central nervous system depressant activity in mice as evidenced by a reduction in locomotor activity and potentiation of pentobarbitone-induced hypnosis. The fractions did not cause neurological deficit or anti-convulsant activity. Both fractions caused reductions in blood pressure and heart rate in rats without showing diuretic activity (Singh *et al.*, 1990b).

Acute toxicity symptoms in mice were effects on the motor activity, on orientation, a reduced reaction to pain and clonic convulsions. Corneal, pedal and pinnal reflexes were affected and the oral LD_{50} of the petrol ethanol extract was 22 g/kg bw. The extract exhibited anti-inflammatory activity in rats and had an analgesic effect in mice. Antipyretic activity required administration at high dosage. No ulcerogenic effect was found on the gastric mucosa of rats (Koley *et al.*, 1994).

The ether soluble fraction of alcohol leaf extract showed good analgesic activity in acute inflammatory pain in rats and mice and it did not show acute toxicity in mice. However, it showed only poor anti-inflammatory activity in rats and it was devoid of anticonvulsant activity (Tandan *et al.*, 1990). At doses higher than 50 mg/kg alcohol extract decreased the blood sugar level. The LD_{50} value in mice was 4.6 g/kg bw (Chattopadhyay, 1999). In rats, paracetamol-induced high serum levels of SGOT, SGPT, acid phosphatase and alkaline phosphatase were lowered after administration of alcohol leaf extract. Liver necrosis and the dilated vasculature and sinusoids around necrotic zones were prevented due to the extract. The extract contains mainly 6-flavanol-O-glycosides which presumably afford protection from the induced liver damage (Chattopadhyay *et al.*, 1992). Alcohol leaf extract in rats lowered serum cholesterol level without altering serum protein, blood urea and uric acid levels (Chattopadhyay *et al.*, 1993b).

Ethanol leaf extract had an anti-inflammatory activity through inhibition of the proliferative phase of inflammation (Chattopadhyay, 1998). Ethanol leaf extract dose-dependently induced mitotic chromosome abnormalities in bone marrow cells of mice. Gross type abnormalities appeared even at the lowest dose and remained unchanged in frequency at higher doses; the individual type abnormalities were induced only at the highest dose (Awasthy *et al.*, 1995). The extract caused increased incidence of structural and mitosis-disruptive changes of metaphase chromosomes. A constituent of the extract, along with free radicals, probably interfered with DNA to yield chromosome strand breakage or produced spindle disturbances, inducing genotoxic effects (Awasthy *et al.*, 1999). Ethanol leaf extract in itself had no effect on peripheral utilisation of glucose, but pre-treatment with the extract

Table 7: Effects of neem extracts made with non-aqueous solvents on animals

Plant part	Solvent	Test animal	Dose (equivalent weight of leaves; fresh (f) or dry (d))	Duration (days)	Observed effect(s)	Reference
ACUTE EFFECTS						
Leaves	Ethanol	Mice	500; 1000; 2000 mg/kg bw (*; d)	7	Genotoxicity	Awasthy *et al.*, 1995
Leaves	Ethanol	Mice	500; 1000; 2000 mg/kg bw (*; d)	7	Genotoxicity	Awasthy *et al.*, 1999
*	*	Mice	2 ml/mouse (*; f)	1	No effect on malaria parasites	Bray *et al.*, 1990
Leaves	Ethanol	Rabbits	200 mg/kg bw (3984; d)	1; 7	Anti-diabetic effect	Chattopadhyay, 1996
Leaves	Ethanol	Rats	100; 200 mg/kg bw (1992, 3984; d)	1; 7	Anti-inflammatory action	Chattopadhyay, 1998
Leaves	Alcohol	Mice, rats	50 - 400 mg/kg bw, 0.25 - 8.0 mg/kg bw (996 - 7968, 5 - 159; d)	1	Anti-diabetic effect	Chattopadhyay, 1999
Leaves	Ethanol	Rats	500 mg/kg bw (*; *)	1	Reduction in hepatic glycogen	Chattopadhyay *et al.*, 1993a
Leaves	Alcohol	Mice, rats	*	1	Effect on blood constituents	Chattopadhyay *et al.*, 1993b
Leaves	Petrol, ethanol	Mice, rats	0.1 - 1.0 g/kg bw, 10 - 40 g/kg bw (3.7 - 37.3, 373 - 1493; f)	1	Anti-inflammatory effect, toxicity	Koley *et al.*, 1994
Seeds	*	Rodents	0.4 ml/animal (*; *)	3	Resorption of embryo's	Mukherjee *et al.*, 1996a
Seeds	Hexane	Rats	25; 50; 75; 100 % (*; *)	3	Abrogation of pregnancy	Mukherjee *et al.*, 1999
Fruits, leaves	Methanol	Rodents	Soaked food (*; d)	5	Anti-feedant effect, toxicity	Oguge *et al.*, 1997
Bark, leaves	Methanol	Mice, rabbits, rats	0.4 - 12.8 g/kg bw (33 - 1052; f)	1	Anti-inflammatory and anti-pyretic effects, toxicity	Okpanyi and Ezeukwu, 1981
Bark	Methanol	Mice	100 mg/kg bw (*; d)	1	Anti-thrombotic effect	Olajide, 1999

Plant part	Solvent	Test animal	Dose	Dura-tion	Observed effect(s)	Reference
Leaves	Acetone	Mice	50; 100; 200 mg/kg bw (*; d)	1	Neuro-psychophar-macological effect	Singh *et al.*, 1987
Leaves	Acetone	Mice, rats	100 mg/kg bw (*; d)	1	Effect on central, autonomic and cardiovascular systems	Singh *et al.*, 1990b
Leaves, seeds	*	Baboon rats	6 ml and 0.6ml/animal (*; *)	3; 6	Effect on reproduction	Talwar *et al.*, 1997
Leaves	Ethanol	Mice, rats	0.1 - 1.0 g/kg bw, 1.3; 10 g/kg bw (16 - 159, 206; 1587; d)	1	Analgesic effect, no acute toxicity	Tandan *et al.*, 1990
SUBACUTE EFFECTS						
Leaves	Ethanol	Rats	100 mg/kg bw (1000; f)	28	Hypolipidaemic effect	Chattopadhyay, 1995
Leaves	Ethanol	Rats	100 mg/kg bw (*; *)	21	Effect on reproduction	Choudhary *et al.*, 1990
Leaves	Chloro-form hexane, methanol	Rats	12.5% in diet (12.5%; f)	14	Anti-mutagenic, anti-carcinogenic effect	Kusamran *et al.*, 1998b
CHRONIC EFFECTS						
Leaves	Alcohol	Mice	500; 1000; 2000 mg/kg bw (*; *)	42	Genotoxicity, sperm deformation	Awasthy, 2001
Bark, flowers, oil	*	Rats	*	*	Reduced male reproduction	Dixit *et al.*, 1992
Husks, seeds	Petroleum ether	Rats	566 mg/kg bw, 360 mg/kg bw (*; f)	60	Effect on blood constituents	Gupta *et al.*, 2001
Husks, kernels	Petroleum ether	Rats	1000 mg/kg, 72 mg/kg bw (*; *)	60	Toxicity	Kataria *et al.*, 2000
*	*	Rats	*	*	Reduced ovarian activity	Mishra, 1996
Flowers	Ethanol	Rabbits	500 mg/kg bw (*; d)	30; 60	Hypolipidaemic effects	Purohit and Daradka, 1999

* = not specified in reference

Hexane seed extract, contradictory to ethanol and water extracts, completely abrogated pregnancy. Restoration of fertility was observed in subsequent cycles and no further toxic effects were found. Treatment with the active fraction of the extract caused specific activation of cell-mediated immune reactions (Mukherjee *et al.*, 1999). Examination in rodents previously treated with seed extracts revealed complete resorption of embryos on day 15 of pregnancy (Mukherjee *et al.*, 1996a). In rats and baboons, treatment with seed extract had no residual permanent effect and fertility was regained in subsequent cycles. Increases in CD4 and CD8 cells were noticed in mesenteric lymph nodes and spleen. A rise in immunoreactive and bioactive TNF-α and IFN-γ in draining lymph nodes, serum and foetal-placental tissue was observed. Moreover, in vitro inhibitory action was found on a wide spectrum of microbes and viruses (Talwar *et al.*, 1997). Leaf extracts did not show anti-malarial activity in vitro or in vivo in mice (Bray *et al.*, 1990).

Neem flowers in the diet of rats increased hepatic GST activity and reduced the activities of P450, aniline hydroxylase and aminopyrine-N-demethylase. The flowers contain phase II enzyme inducers and compounds capable of repressing monooxygenases, especially those involved in metabolic activation of chemical carcinogens (Kusamran *et al.*, 1998a). Leaf extracts contain a weak antimutagen, as was revealed in an Ames' test. The mechanism of the anti-mutagenicity may be through inhibition of the activity of metabolic-activating enzymes in the liver (Kusamran *et al.*, 1998b). Treatment of rats with ethanol leaf extract reduced elevated serum levels of cholesterol, total lipids and triglycerides (Chattopadhyay, 1995). Ethanol leaf extract did not interfere with spermatogenesis, but anti-implantational and abortifacient effects were observed in females mated by treated males (Choudhary *et al.*, 1990).

Rats receiving 72 mg/kg husk extract died within 20 days of treatment. Toxicity signs were laboured respiration, strub tail, salivation and analgesia. Prolonged administration of kernel extract caused a decrease in Hb, mean corpuscular Hb concentration and lipid peroxidation, an increase in the white blood cell count and in blood GSH content, and inhibition of erythrocyte catalase. An increase in blood urea nitrogen was observed in treated animals, whereas serum amylase increased for kernel extract and decreased for husk extract. Both extracts decreased in SGOT and SGPT, but there was no effect on total protein content. Plasma total lipids increased, as did serum cholesterol concentration for husk extract and plasma phospholipids for kernel extract. However, husk extract decreased plasma phospholipids and blood glucose levels. Husk extract was more toxic than kernel extract (Kataria *et al.*, 2000). A decrease in TEC and in ESR and an increase in mean corpuscular volume were observed after administration of seed or husk extract to rats. No changes in Hb, PCV, TLC, mean corpuscular Hb concentration and the blood glucose level were found due to treatments, but blood urea nitrogen was increased and SGOT and SGPT decreased. Serum

protein, serum cholesterol, plasma total lipids and GST increased, while plasma phospholipids and erythrocyte acetylcholinesterase decreased (Gupta *et al.*, 2001). After administration of ethanol flower extract to rabbits, high cholesterol levels were ameliorated and phospholipid and triglyceride levels reduced. Tissue lipid profiles of liver and heart muscle showed changes similar to those noticed in serum lipids (Purohit and Daradka, 1999). Neem extract was active against some fungi, bacteria, and viruses. Cholesterol and triglycerides levels in blood serum and liver decreased in rats (Sharma *et al.*, 1999).
Kumar and Jattan (1995), in an overview of literature, reported the contraceptive activity of extracts in male and female rats and mice after different kinds of administration. Mishra (1996) reported that extract reduced the weight of ovaries and uterus of treated rats. Contents of ascorbic acid and cholesterol of ovaries increased. Extracts of bark, flowers and seed oil induced reversible infertility in male rats. This was measured as decreases in the spermatid number, the mature Leydig cell population and the cauda sperm count. Testicular protein, sialic acid, glycogen and vesicular fructose levels were reduced. Neem oil reduced blood glucose levels (Dixit *et al.*, 1992). As reported from shorter term tests, upon chronic administration ethanol leaf extract in mice increased incidences of structural changes and synaptic-disturbances in meiotic chromosomes and it caused disruptions of meiosis. The extract reduced the sperm count and increased the frequency of spermatozoa with abnormal head morphology (Awasthy, 2001).

5.3 Conclusion

The negative effects of non-aqueous neem extracts generally can be summarised as toxic and genotoxic effects and as effects on male and female reproductive ability.
Awasthy *et al.* (1995; 1999) found that 2 g/kg bw had adverse effects in all parameters of genotoxicity they measured, whereas 1 g/kg bw did not cause individual type abnormalities or structural changes. The authors used an ethanol extract from dried leaves. The yield of extract from one kilo dry leaves ranges from 6.3 g (Tandan *et al.*, 1990) to 50.2 g (Chattopadhyay, 1996, 1998, 1999). This would mean that the NOAEL of 1 g extract/kg bw in mice would be equivalent to 20 - 159 g dry leaves/kg bw. For an adult human this would mean that ingestion of the non-aqueous extract of as much as (0.1 * 0.1 * 70 * 20 - 159 g) 14 - 111 g dry leaves would be safe. Obviously, the toxic compounds that are present in the unprocessed leaves are not all extracted with the non-aqueous solvents.
Singh *et al.* (1987) found an ED_{50} value for impairment of the motor coordination of 30 mg acetone leaf extract/kg in mice upon acute exposure. If a yield of 50 g/kg dry leaves is presumed, this dose would be the equivalent of 600 mg dry leaves/kg. This implies that a calculated safe dose for daily human exposure should be lower than 0.3 mg acetone extract/kg bw, which is equivalent to less than 6 mg dry leaves/kg bw. The value of 0.08 mg/kg calculated for safe daily exposure to unprocessed leaves is indeed below this level.

Mukherjee *et al.* (1999) reported effects for administration of a pure extract, but not for the same extract diluted to 75%. However, since no volumes or weights are given in the article, no conversion can be made and no NAEL can be calculated. For the doses used by Koley *et al.* (1994) for evaluation of the toxicity, the effects are too severe to extrapolate and calculate a reliable NAEL.

6 Pure compounds

Pesticide legislation requires detailed information on the exact ingredients of the products. However, for neem the active compounds and their concentration differ with the plant parts used and with their age, growing situation etc. (Schoonhoven *et al.*, 1998). Many of the secondary compounds of neem have been identified (Van der Nat et al, 1991), purified and some have been tested for their effects on mammals. These include for example azadirachtin, nimbolide and nimbinin.

6.1. Effects on humans

Effects of pure neem derived compounds on human health are not documented. Beard (1989) considered that azadirachtin was not toxic to humans, but when it was administered to the kissing bug (*Rhodnius prolixus*) the agent inhibited the development of the bug itself and of the parasitic flagellate *Trypanosoma cruzi* inside the bug. With the inhibition of the vector and the infective flagellate, chagas disease could be prevented.

6.2 Effects on animals

In Table 8, documentation of effects of pure neem-derived compounds on animals is summarised. When rats were treated with azadirachtin, increased serum SGOT and SGPT activities and bilirubin content were observed. Histopathological studies showed pathological changes in the liver in terms of congestion, hydropic degeneration, necrosis and lymphocytic infiltration (Abdel Megeed *et al.*, 2001).
In acute cases, nimbidin, isolated from seeds, dose-dependently reduced acute paw oedema in rats, and suppressed induced arthritis and fluid exudation in induced granuloma. The ED_{50} value was 79.4 mg/kg in rats (Pillai and Santhakumari, 1981).
The limonoids nimbolide and nimbinin showed in vitro activity against *Plasmodium berghei*. Nimbolide in mice had an ED_{50} value of 135 mg/kg/day (Bray *et al.*, 1985), but in vivo no anti-malarial activity was observed (Bray *et al.*, 1990).

In rats, azadirachtin caused stimulation of the albumin content, an increase of the blood glucose level and protein content. The red blood cell content was not affected, but the white blood cell content and platelet counts increased (Radwan *et al.*, 2001a).

After rats had been administered azadirachtin during pregnancy (days 6 - 15) no adverse embryo/foetotoxicity and teratogenic effects or effects in reproductive parameters were found. The total number of implantations, post-implantation loss and foetal weight were not altered and there were no malformations due to the treatment (Srivastava and Raizada, 2001).

Table 8: Effects of pure compounds of the neem tree on animals

Compound	**Test animal**	**Dose**	**Duration (days)**	**Observed effect(s)**	**Reference**
ACUTE EFFECTS					
Azadirachtin	Rats	0.1 LD_{50} = 57 ppm	1 - 3	Effect on liver function	Abdel Megeed *et al.*, 2001
Limonoids	Mice	135 mg/kg bw	4	No anti-malarial effect	Bray *et al.*, 1985
Limonoids	Mice	2 ml/mouse	1	Effect against intestinal parasites	Bray *et al.*, 1990
Nimbidin	Rats	20; 30; 40 mg/kg bw	1	Anti-arthritic and anti-inflammatory effect	Pillai and Santhakumari, 1981
SUBACUTE EFFECTS					
Nimbidin	Dogs, guinea pigs, rats,	20 – 80 mg/kg bw	1, 10, 28	Anti-ulcer effect	Pillai and Santhakumari, 1984a
Azadirachtin	Rats	0.1 LD_{50}	21	Effect on blood constituents	Radwan *et al.*, 2001a
Azadirachtin	Rats	0.5; 1.0; 1.5 g/kg bw	21	No foetotoxicity or teratogenicity	Srivastava and Raizada, 2001
CHRONIC EFFECTS					
Azadirachtin	Rats	0.5; 1.5; 4.5 ml/kg bw	60	Effect on liver and haemopoietic system	Gupta *et al.*, 1998
Nimbidin	Dogs, mice, rats	20 – 2000 mg/kg bw, 25 - 100 mg/kg bw, 10; 20 mg/kg bw	1, 42, 28	No toxicity	Pillai and Santhakumari, 1984b
Azadirachtin	Rats	0.1 LD_{50}	42	Effect on blood constituents	Radwan *et al.*, 2001b
Azadirachtin	Rats	5 g/kg bw, 0.5 – 1.5 g/kg bw	1, 90	No toxicity	Raizada *et al.*, 2001

When rats were administered azadirachtin at high dose, a decrease in body weight gain and relative liver weights was observed. Serum protein, albumin and creatinine as well as TEC, Hb, ESR, PCV and TLC were lowered, SGOT increased, but no effect was found on blood urea nitrogen and SGPT. Histopathologically, non-specific generalised degenerative changes were found. Thus, the formulation led to adverse effects on the haemopoietic system (Gupta *et al.*, 1998). Azadirachtin in rats caused an increase of the blood urea content and in uric acid followed by a decrease to the normal rate. Residues of the compound caused tubular degradation and lesions (Radwan *et al.*, 2001b). There was no acute toxicity of azadirachtin in rats even at 5 g/kg. At lower doses for a longer period, the formulation caused aggressiveness in a dose- and time-dependent manner, but no signs of toxicity were seen. Body weight, vital organs, enzyme activities in liver and serum and blood parameters did not change due to the treatment (Raizada *et al.*, 2001).

Subacutely, nimbidin provided a protective effect against ulcers in induced gastric and duodenal lesions in rats and guinea pigs. It enhanced the healing process in acetic acid-induced chronic gastric lesions in rats and dogs (Pillai and Santhakumari, 1984a).

Administration of nimbidin to rats, mice and dogs did not produce any signs of toxicity, although a dose-related weight gain, an increase in Hb level, an increase in liver glycogen and a reduction in serum protein were observed. The LD_{50} value was higher than 2 g/kg. Teratogenic studies in rats did not reveal any toxic manifestations or foetal abnormalities (Pillai and Santhakumari, 1984b).

6.3 Conclusion

The best-documented pure compound from the neem tree is azadirachtin, which is present in leaves at an estimated average concentration of 1.5 g/kg (Oguge *et al.*, 1997) and in kernels up to 9 g/kg (Ascher, 1993). From one kg kernels, 100 ml oil can be extracted by hand (Anonymous, 1995) and other extraction methods yield even 200 g oil/kg kernels (Saxena, 1989). If azadirachtin is retained in the oil and is completely extracted, the concentration would be up to 45 - 90 g azadirachtin/kg oil.

Upon subchronic administration, measuring effects on rats' foetuses (Srivastava and Raizada, 2001) or when administered chronically, a dose of 1.5 g azadirachtin/kg bw in rats had no adverse effects (Gupta *et al.*, 1998; Raizada *et al.*, 2001). This NOAEL could be translated to a safe chronic dose for human consumption of 0.1 * 0.1 * 1.5 g = 0.015 g azadirachtin/kg bw.

Furthermore, taking into account that a LOAEL of 2.0 ml oil/kg was reported for the anti-fertility effect of neem oil in female rats, and that neem oil is known to contain about 45 - 90 g azadirachtin/kg, it can be calculated that ingestion of 2.0 ml oil/kg implies exposure to 0.09 - 0.18 g azadirachtin/kg bw. This is far below the reported NOAEL of 1.5 g azadirachtin/kg bw. This leads to the conclusion that the toxic effects of neem oil are unlikely to be caused by its azadirachtin content.

7 Neem-based commercial products

In some countries, selling neem-based products or pesticides on the market is allowed. These include products like Praneem, a purified seed extract, and Ectozee.

7.1. Effects on humans

Little is published about the effect of such products on humans. Only for Praneem, a purified seed extract, some studies were published as summarised in Table 9. A Praneem cream was developed which was devoid of irritation and sensitisation potential, as tested on rabbits and in a 21-day test on skin sensitivity in human volunteers. Subacute toxicity studies with monkeys indicated that the formulation was safe. The cream had a high contraceptive efficacy in rabbits and monkeys after intravaginal application (Garg *et al.*, 1993b). The minimum effective spermaticidal concentration for Praneem was 25%. At this concentration, 100% of the sperm were immobilised within 20 seconds (Garg *et al.*, 1994). The toxicity aspects of Praneem as a contraceptive were investigated by Talwar *et al.* (1995). There were no immediate or delayed reactions to the treatment. Haematological and biochemical parameters stayed within normal limits.

Table 9: Effects of neem based products on humans

Product	Administration	Dose	Duration	Observed effect(s)	Reference
Praneem	Topical	0.5 ml/d	21 days	No skin sensitivity	Garg *et al.*, 1993b
Praneem	In vitro	5 µl/µl sperm	Once	Spermaticidal effect	Garg *et al.*, 1994
Praneem Vilci	Intra-uterine	1.5; 2; 2.5; 3 ml	3 days	No toxicity	Talwar *et al.*, 1995

7.2 Effects on animals

The results of studies on effects of neem-based products and pesticides on animals are summarised in Table 10. Oral treatment with Praneem was well tolerated by pregnant baboons and bonnet monkeys, but pregnancy was terminated due to the treatment. No behavioural changes or alteration in food intake were observed. Blood biochemistry and liver function were not altered and the treated animals regained normal cyclicity and gave birth to normal offspring later (Mukherjee *et al.*, 1996b). Administration of Praneem to pregnant rats caused complete resorption of the developing embryos on day 15 of pregnancy. The effect of the treatment was reversible and animals regained fertility. On administration, serum levels of T_H1 cytokines (γ-interferon and tumour necrosis factor) were raised, which may be the cause of pregnancy termination (Mukherjee and Talwar, 1996).

In rats, high doses of Ectozee, a herbal product containing extracts of neem, led to anorexia, enlargement of the abdomen, drowsiness, tetanic spasms and haemorrhagic diarrhoea mostly resulting in death. At 100% concentration the product was highly toxic and led to bleeding from the mouth, mucohaemorrhagic diarrhoea and bulging of eyeballs. Surviving rats entered a state of tetanus and then coma. Post mortem evaluation revealed haemorrhages in the gastrointestinal tract, liver and lungs, the severity of which was proportional to the ingested concentration of Ectozee (Das, 1999).

After treatment of anorectic goats with a neem-containing herbal preparation called Ruchamax, their appetite was restored. The rumen motility and the total bacterial and protozoal counts increased after treatment (Phalphale *et al.*, 1997). Hepatogard, a herbal preparation containing 10% neem, exhibited hepatoprotective activity, and it reversed CCl_4 induced biochemical and histopathological changes. Serum levels of SGOT, SGPT, albumin and total protein were lowered due to the treatment (Rao *et al.*, 1993).

Table 10: Effects of neem based products on animals

Product	**Test animal**	**Dose**	**Duration (days)**	**Observed effect(s)**	**Reference**
ACUTE EFFECTS					
Ectozee	Rats	0.1 - 0.5 ml/rat	1	Toxicity	Das, 1999
Praneem	Rats	0.6 ml/rat	3	Abortive effect	Mukherjee and Talwar, 1996
Praneem	Baboons, monkeys	3; 6 ml/animal	6	Embryo resorption	Mukherjee *et al.*, 1996b
Hepatogard	Rats	650 mg/kg bw	1	Hepatoprotective	Rao *et al.*, 1993
SUBACUTE EFFECTS					
Nimbokil-60	Mice, rabbits	1 - 10 ml/kg bw, 0.0025; 0.005; 0.01 ml/kg bw	1, 28	Acute and subacute toxicity, effect on fertility	Kazmi *et al.*, 2001
CHRONIC EFFECTS					
Tric Vet Care	Rats	0.5; 1.5; 4.5 ml/kg bw	60	Effect on blood constituents, effects in organs	Kataria *et al.*, 1998
Vepacide-Tech	Rats	80; 160; 320 mg/kg bw	90	Toxicity	Mahboob *et al.*, 1995
Vepacide-Tech	Rats	1.0; 1.5; 2.0 g/kg bw, 80; 160; 320 mg/kg bw	1, 90	Effects in organs, toxicity	Mahboob *et al.*, 1998
Vepacide	Rats	80; 160; 320 mg/kg bw	90	Effect on enzyme profiles	Rahman *et al.*, 2001

At low doses, Nimbokil did not show adverse effects on mice and it did not reduce their fertility. The LD_{50} value was 16 ml/kg bw and the LD_{100} value was 20 ml/kg bw. Upon autopsy, no gross changes were seen in heart, lungs, liver, kidneys, ovary and testicles of the test animals. However, the product had a depressant effect on the central nervous system which, at higher concentrations, resulted in death (Kazmi *et al.*, 2001).

Long-term administration of Vepacide to rats caused dose-dependent loss in body weight and food intake, dullness, irritation, diarrhoea and weakness. Biochemical studies showed a dose- and time-dependent increase of SGPT and SGOT levels in serum, kidney and lung while these enzymes decreased in the liver. This profile indicates necrosis of the liver, and an adaptive mechanism in the other tissues due to the chemical stress. Lungs, liver and kidneys were most affected by the treatment (Rahman *et al.*, 2001). Acute administration of 80 - 320 mg/kg bw Vepacide-Tech (12% azadirachtin) in rats resulted in 10 to 80% mortality. Upon chronic administration, the highest doses caused a decrease in the P450 concentration in liver and lungs and all doses affected the kidneys. Cyt.b5 concentration decreased in brain, liver, lungs and kidneys at high doses. P450 reductase concentration decreased in liver and brain at high doses (Mahboob *et al.*, 1998). The oral LD_{50} in rats was reported to be 1.6 g/kg, indicating that Vepacide-Tech is moderately toxic to rats. When given in lower doses for a longer period, the product induced time-, dose- and tissue-specific inhibition in GST, GSH and UDP-glucuronyltransferase activity in liver, lungs, kidneys and brain. Higher doses caused alterations in the incidence of detoxification enzymes of various tissues. The changes induced by Vepacide were reversible on cessation of the treatment (Mahboob *et al.*, 1995).
In rats treated with Tric Vet Care, catalase activity of the red blood cells increased. At high doses, lipid peroxidation increased in the brain and total ATPases decreased in both brain and liver. The activity of Mg^{2+} ATPase and acetylcholinesterase increased in the liver while they decreased in the brain. The product affected liver and brain functions, possibly through membrane alteration and it could influence the oxidant defence mechanism of red blood cells and brain (Kataria *et al.*, 1998).
In a review on the health evaluation of NeemazalTM-T/S, this product was found not to cause any effect on reproduction and it did not cause skin or eye irritation. LD_{50} values in several test animals were higher than 2 g/kg. No carcinogenicity was observed and a no-effect level of 100 ppm was determined upon 90 day administration in rats (Niemann and Hilbig, 2000).

7.3 Conclusion

Most of the neem-based products are toxic. For Praneem and Nimbokil-60 effects on reproduction and fertility are reported. Ruchamax and Hepatogard were even reported to have positive effects in short term studies. All other agents, administered once or

chronically, negatively influenced animal health and in some cases even caused death with LC_{50} values varying in the range from 1.6 g/kg bw to 16 ml/kg. The chemical composition of the commercially available agents is not always stated in the publications and the safe levels of ingestion as compared to the crude neem products and/or the various neem-based extracts and oils cannot be estimated.

8 Toxicity of neem as an insecticide

For the negative effects measured as a consequence of treatments, the NOAEL or, if that is not available from the references the LOAEL for unprocessed neem material, neem oil, aqueous and non-aqueous neem extracts and pure neem compounds are summarised in table 11. There is no obvious trend for the values for the period of administration; chronic NOAEL's are not always lower than acute ones. The oil and the pure compounds are less toxic than unprocessed material or extracts. From this summary, it appears that aqueous extracts are roughly as toxic as non-aqueous extracts.

For all preparation methods, both toxic and beneficial effects were reported for which the applied dose does not seem the distinguishing factor; in some cases, the toxic effects are found at lower doses than the positive effects. The amount of active ingredients in the preparation might be influenced by the origin of the neem material used (Ascher, 1993), or the time between preparing and applying the agent. For calculations on the risk of pesticide residues, the effects of the period of storage itself and of high temperatures during cooking of treated products on the active ingredients of neem are still to be examined. Therefore, the calculations presented here cannot be precise and safety should be better investigated, but the calculated safe doses give at least an indication of the risk one might run upon ingestion of neem or neem-treated products.

The ingestion of neem products as pesticide residues on beans can be compared to the risk assessments made. A generally used dose of neem leaf powder for the protection of stored beans against insects is 25 g/kg seeds (See Part I of this thesis for an overview). A daily meal of 150 g beans, presuming no effect of washing, would then contain maximally 3.75 g of powder residue. This dose is about 670 times higher than the calculated safe dose of 5.6 mg in section 2.3. However, most of the powder is easily sieved off or removed after washing of the treated seeds with water.
Neem oil is generally applied at about 5 ml/kg beans. In a meal of 150 g beans this would leave a residue of 0.75 ml. This is about five times higher than the estimated safe dose of 0.14 ml oil calculated in section 3.3, but it is lower than the doses of 5 ml shown to be toxic upon ingestion by small children (section 3.1).

Table 11: Overview of NOAELs or LOAELs for unprocessed neem material, neem oil, aqueous and non-aqueous neem extracts and pure neem compounds.

	Reference	Effect	Animal	Dose	Parameters measured
Unprocessed material					
Acute	Jaiswal *et al.*, 1994	LOAEL	Rats	10 mg/kg	Anxiolytic activity
Subacute	Kasturi *et al.*, 1995, 1997	LOAEL	Rats	80 mg/kg	Male fertility
	Parveen et al, 1993	LOAEL	Rats	80 mg/kg	Male fertility
Chronic	Aladakatti *et al.*, 2001	LOAEL	Rats	500 mg/kg	Male fertility
Oil					
Acute	Gandhi *et al.*, 1988	NOAEL	Rabbits, rats	5 ml/kg	Toxicity
	Tandan *et al.*, 1995	NOAEL	Mice	7.4 g/kg	Toxicity
Subacute	Dhaliwal *et al.*, 1998	LOAEL	Rats	2 ml/kg	Female fertility
Chronic	Chinnasamy *et al.*, 1993	NOAEL	Rats	10 g/kg	Toxicity
Aqueous extract					
Acute	El-Hawary and Kholief, 1990	LOAEL	Rats	200 mg/kg	Toxicity and anti-diabetic effect
Subacute	Panda and Kar, 2000	NOAEL	Mice	40 mg/kg	Adverse effect on thyroid function
	Ray *et al.*, 1996	NOAEL	Mice	30 mg/kg	Immune response
Chronic	Ali, 1987	LOAEL	Goats, guinea pigs	50 mg/kg	Toxicity
Non-aqueous extract					
Acute	Singh *et al.*, 1987	LOAEL	Mice	50 mg/kg	Neuropsychopharma-cological effect
Subacute	Choudhary *et al.*, 1990	LOAEL	Rats	100 mg/kg	Effect on reproduction
Chronic	Kataria et el., 2000	LOAEL	Rats	72 mg/kg	Toxicity
Pure compound					
Acute	Abdel Megeed *et al.*, 2001	LOAEL	Rats	57 ppm	Effect on liver function
Subacute	Srivastava and Raizada, 2001	NOAEL	Rats	1.5 g/kg	Foetotoxicity, teratogenicity
Chronic	Raizada *et al.*, 2001	NOAEL	Rats	1.5 g/kg	Toxicity

The effects of aqueous extracts on animals are ambiguous, as is their use on stored seeds, because stored seeds should be kept as dry as possible to prevent moulds and early germination (De Groot, 1996). No safe dose can be proposed here.

For non-aqueous extracts, once the solvent has evaporated, the doses of neem material on the seeds will be low. However, the exact dose is difficult to estimate. No reliable estimations can be made on the risks caused by these residues.
Pure neem compounds, especially azadirachtin, when calculated relative to the quantities of crude material, could be called non-toxic. For neem-based pesticides, a range of tests should be performed before their commercial release. The effects and safe use and dose should be mentioned on the packages.
From the rough risk assessments presented in this study, the use of neem based products as insecticides to protect stored seeds for consumption, if applied with care, should not be discouraged. At the indicated doses, most of the neem preparations are effective against insects and the advantages of keeping the stored seeds in a good condition at low cost would outweigh the disadvantageous effects of the treatment.

Acknowledgements
The research was financed by the Centre Béninois pour le Développement Durable. SJB wishes to thank J.E. Boeke and H. Feitsma for their help supplying the articles.

Abbreviations
bw: body weight
DMBA: 7,12-dimethylbenz[α]anthracene (carcinogenic agent)
Cyt.b5: cytochrome b5
ED_{50}: Medium effective dose
ESR: erythrocyte sedimentation rate
GGT: gamma glutamyl transpeptidase
GPx: glutathione peroxidase
GSH: glutathione
GST: glutathione-S-transferase
Hb: haemoglobin
LD_{50}: medium lethal dose
L(O)AEL: lowest (observed) adverse effect level
N(O)AEL: no (observed) adverse effect level
P450: cytochrome P450
PCV: packed cell volume [haematocrit]
SGOT: aspartate aminotransferase [glutamic oxaloacetate transaminase]
SGPT: alanine aminotransferase [glutamic pyruvate transaminase]
TEC: total erythrocyte count
TLC: total leukocyte count

Chapter 7

Field trials with plant products to protect stored cowpea against insect damage

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Abstract

Plant products were evaluated under field conditions for their efficacy as insecticides against the cowpea beetle, *Callosobruchus maculatus* on stored cowpea. Seeds, mixed with finely ground clay and three volatile oils were stored in air-tight jerry-cans and canisters. Pods were treated with leaf powders of two plant species and stored in traditional palm-leaf huts. Beetle damage was evaluated before and after storage. The treatments did not prevent damage, but after treatment with oils, fewer beans showed beetle emergence holes and the percentage of uninfested beans and the weight of one litre of beans were higher than for untreated beans. The percentage of germination of stored beans was highest after treatment with *Ocimum basilicum* oil. Leaf powder of *Momordica charantia* was effective against weight loss of stored seeds, whereas *Ficus exasperata* caused a decrease in both the percentage of infested beans and the number of emerged beetles and more parasitoids emerged than from untreated beans. Laboratory tests on the effect of the oils on the development of the beetle and on bean germination did not reveal effects of the oils. A comparison between data obtained from the laboratory and those obtained in the field is made.

Key words: *Callosobruchus maculatus*, botanical insecticides, volatile oils, traditional storage

Introduction

In the laboratory and in semi-field tests, much research has been done to evaluate the use of plants as insecticides for the protection of stored seeds against beetle pests (reviewed in Part I of this theses). The results are often promising and the insecticidal plants could offer a cheap, safe and environmentally sound alternative for synthetic insecticides.
However, the results obtained in the laboratory are hardly if ever verified in a field situation and the results from (semi-) field tests are mostly not linked to those from laboratory tests.

In the laboratory, the mechanism of action of insecticidal plants and the most susceptible developmental stage of the insect pest are often investigated. This way the effects of the botanical insecticide can be determined under optimised conditions. Field tests mostly report the condition of the beans after a certain period of storage without reporting the status of the pest insect population. Therefore, the results of both types of tests are difficult to compare and the two separate ways of research each produce data that are hardly ever linked.
The use of plants as insecticides on stored seeds is often an age-old practice. In West Africa, cowpea, *Vigna unguiculata* (L.) Walp. was traditionally treated with dried ground plant material to protect the beans against the cowpea beetle *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae). With the introduction of synthetic pesticides, much of the knowledge on these practices was forgotten (Atteh, 1984), but previous research in the laboratory on such traditionally used plants showed promising results for successful application of some plants to protect stored cowpea seeds (Chapter 3 & 4).
To check if the results obtained in the laboratory are comparable to results from trials carried out in a field situation, we tested plant products from Benin in storage structures as farmers traditionally use them. The damage and side effects on germination and water absorption were investigated.

Material and methods

All storage tests were conducted from April to December 2001 under ambient conditions in the south of Benin. The average temperature was 27 ± 5°C and the relative humidity ranged from 60-80 %. In the laboratory and incubation rooms, the average temperature was 30 ±1 °C with the same humidity and a photoperiod of 12:12h.

Storage as grains with ground clay and volatile oils, Dannou

Three volatile oils extracted from plants occurring in West Africa were purchased in supermarkets in Benin. The oils had been distilled from *Clausena anisata* (Willd.) Hook ex f. Benth., (Rutaceae), *Ocimum basilicum* L. (Lamiaceae) and from a mixture of *Cymbopogon citratus* (DC ex Nees) Stapf, and *Cymbopogon flexuosus* Stapf & J.F. Watson, (Poaceae) (further referred to as *Cymbopogon* MIX).
Ten farmers from the Dannou village in the Ouémé valley each sold 20 kg of cowpea seeds of the local variety Chaoué to be treated with plant products. The beans of this variety are large and white, with a rough testa and a small dark eye. The beans had not been treated after harvest in February and at the time of treatment, in April, they were already naturally infested with bruchid beetles. At least two species of parasitic wasps, an egg parasitoid and a larval parasitoid were present on the beans as well. Both parasitoid species lay their egg in the developing beetle and develop at the expense of their host.

The total 200-kg batch of seeds was first mixed thoroughly to guarantee a homogeneous stock of beans. A random sample of 500 g seeds was taken to the laboratory to determine the initial infestation rate.

The seeds were stored in 10 l black plastic jerry-cans and 5 l canisters with transparent plastic lids. First the untreated control containers were filled to the rim with cowpea seeds and closed. Clay treated controls, with finely ground dry river clay mixed at 40 g/kg with the beans were then prepared. For the treatment containers, the same amount of clay was mixed with 0.1 ml/kg volatile oil and this mixture was thoroughly mixed by hand with the beans. The clay was used to facilitate the even mixing of the very small volume of oil with the beans. The mixture of oil, clay and beans was put in the container, which was firmly closed.

In total 11 jerry cans were filled with three replications per oil treatment, one untreated control and one clay treated control and 14 canisters with four repetitions per plant oil and the two controls. The containers were randomly assigned to the farmers and stored in their houses in the shadow under ambient conditions.

To evaluate the initial damage, in the laboratory, the 500-g seed sample was divided into portions of 30 g after removal of all live insects. For ten of these 30 g portions, the number of clean beans, of beans bearing bruchid eggs, with beetle emergence holes and with signs of parasitism (eggs turn black upon parasitisation, larval parasitoids emerge from the beetle larva inside the bean via a characteristic emergence hole) were counted. The moisture content of the seeds was determined by weighing 10 g of seeds and weighing them again after they were dried in a stove at 105°C for 72 hours. Six replicates were measured.

At December 5, after 236 days of storage, the stored containers were opened. From each container, a sample of 500 g, collected according to the coning method (Golob, 1976), was taken to the laboratory for evaluation of the bruchid damage. From the control containers four sub-samples were taken for the canisters and three for the jerry-cans. These sub-samples were statistically treated as repetitions. To evaluate the damage, measurements were done on the percentage of beans without bruchid infestation, with bruchid eggs, with bruchid emergence holes, with signs of parasitism. The number of beans in 10 g, the weight of one litre of seeds, the moisture content and the water absorption of the seeds, and the seed germination were established as well.

The water absorption, an indication for cooking properties of the beans, was defined as the quantity of cold water absorbed by 10 g seeds after 30 minutes: Absorption = ((50 ml – remaining water)/ 50 ml)*100. Four repetitions were done per 500 g sample. For the germination, 20 randomly selected undamaged seeds were put on filter paper in a petri dish and sprinkled with water once a day. After five days, the percentage germination was calculated.

The data were analysed according to a general linear model and LSD post-hoc tests were performed.

Storage as pods with plant powders, Igana

In November 2000, leaves of *Ficus exasperata* Vahl (Moraceae) and *Momordica charantia* L. (Cucurbitaceae) were collected in Benin and dried in the shade under ambient conditions. The dry material was ground with a pestle and mortar. The obtained powders were sieved through a 450 µm sieve and stored at 4°C before use.

In Igana on the plateau in the Ouémé province, the most cultivated cowpea variety is Delekinwa, with small, light brown seeds with a smooth testa, which is generally stored in the pod. From preliminary counting it appeared that one kg of pods contained 600 g of grains. For our research, nine farmers offered a granary and a bag with 5 kg cowpea of this variety in the pod for storage with plant powders. The beans had been harvested in February 2001 and had been left untreated since.

Of the pods in each bag, a sample of 200 g was taken to investigate the initial infestation. The remainders of the samples were each put in a traditional palm-leaf hut on a wooden support. These granaries were positioned around the village between the houses and the borders of the local flora and they were all separated by at least 200 m. The plant powders were applied in three layers between layers of pods at 25 g/kg seeds, thus at 75 g/ per sample of 5 kg pods. The huts were carefully closed with palm leaf shields. In total, there were nine granaries: four treated with *Ficus exasperata* Vahl, Moraceae, four treated with *Momordica charantia* Lam., Moringaceae and one untreated control.

In the laboratory, from each of the nine 200-g samples of pods, forty pods were randomly picked and evaluated for the external signs of insect infestation. The seeds were then collected from these pods, weighed and evaluated for the numbers of damaged and undamaged seeds and the numbers of insects on the beans.

Samples of 40 pods and of one kg of pods per storage hut were taken at the end of the storage period, on September 17, after 156 days. From the control structure, four sub-samples were taken according to the coning method (Golob, 1976). These sub-samples were statistically treated as repetitions. The level of damage was evaluated measuring the weight of the pods and of the healthy and infested beans inside them, the weights of healthy and infested beans, and the numbers of bruchids and parasitoid wasps.

The data were subjected to a general linear model and LSD post-hoc tests were performed.

Laboratory assays for the effect of oils on *Callosobruchus maculatus*

The effects of the essential oils used in the field test, *Clausena anisata*, *Ocimum basilicum* and *Cymbopogon* MIX on the development of *C. maculatus* were tested in a laboratory set up. In a petri dish, 40 g of uninfested cowpea of the variety Californian Blackeye, which is susceptible to bruchids (Baker *et al.*, 1989), was either left untreated or treated with ground clay (40 g/kg) alone or mixed with volatile oil (0.5 ml/kg beans). Two males and one female *C. maculatus* beetle, newly emerged from the laboratory rearing were released on these beans. The number of beetle eggs was counted after 24 hours and the longevity of the

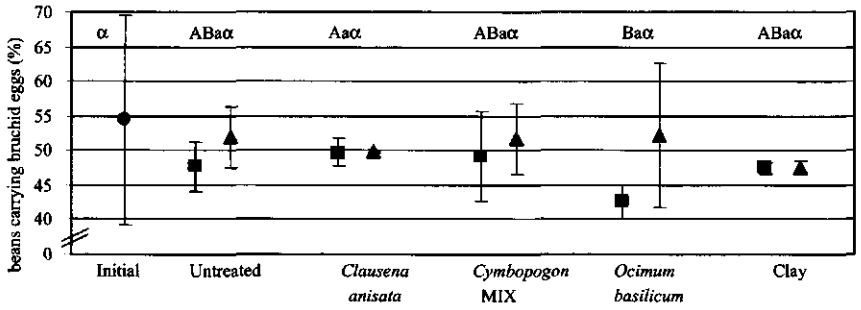
beetles was noted in days. After the death of the beetles, the total number of eggs was counted and the emerging offspring was sexed and removed from the dish within 24 h after emergence.
The data of five repetitions were analysed with a multivariate general linear model and LSD post-hoc tests were performed.

Laboratory assay for the effect of oils on bean germination
In a petri dish, 40 g clean uninfested cowpea beans of the variety Californian Blackeye was treated with 20 µl oil dissolved in 1 ml ethanol. An ethanol-treated and an untreated control were prepared as well. After 15 minutes evaporation of the ethanol, 25 beans per treatment were put in a 9-cm petri dish with two sheets of filter paper wetted with 10 ml of distilled water. After 72 hours of incubation, the number of germinated beans was counted. The same was done with another set of beans 14 days after treatment. These beans were stored at 30°C to imitate storage conditions. Four replicates (100 beans in total) were observed per treatment. The data were submitted to ANOVA in a completely random design and the means were compared with an LSD post-hoc test.

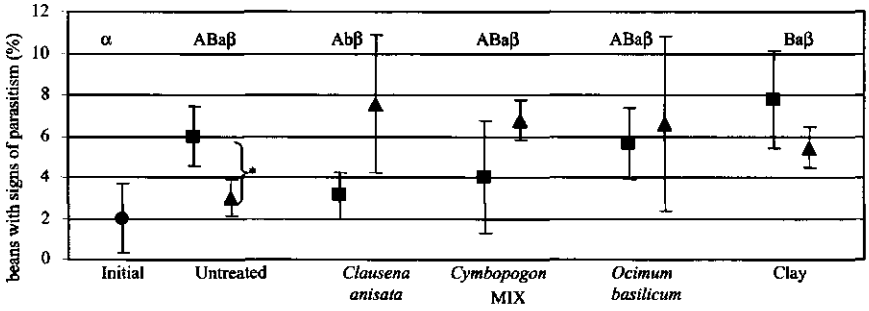
Results

Storage as grains with ground clay and volatile oils, Dannou
After storage, the damage was evaluated and, if the parameter had been measured before storage, it was compared to the initial damage level as shown in figure 1.
The percentage of beans carrying eggs of *Callosobruchus maculatus* without other signs of infestation had not changed after the storage period and was similar for treated and untreated beans. More beans carried eggs in the *Clausena anisata* treated jerry-cans than in the *Ocimum basilicum* treated jerry-cans. There was no difference between the two types of containers concerning the percentage of beans carrying beetle eggs.
The percentage of beans showing signs of parasitisation (i.e. beetle eggs turned black or parasitoid emergence holes were visible) was higher after storage than initially for all vessels. After storage, the percentage of seeds showing parasitisation in canisters was higher on beans treated with *Clausena anisata* than on all other beans. For the jerry-can samples, the percentage was higher on clay treated control beans than on *Clausena anisata* treated beans. When the two container types were compared, the percentage of beans showing signs of parasitism was higher in the untreated jerry-can than in the untreated canister, while for non of the treatments a significant difference was found.
After storage, the percentage beans with bruchid emergence holes was higher than initially for all stored samples. The percentage was higher for the untreated control than for any of the oil treated bean samples. In the oil treated jerry-cans fewer beans had bruchid holes than

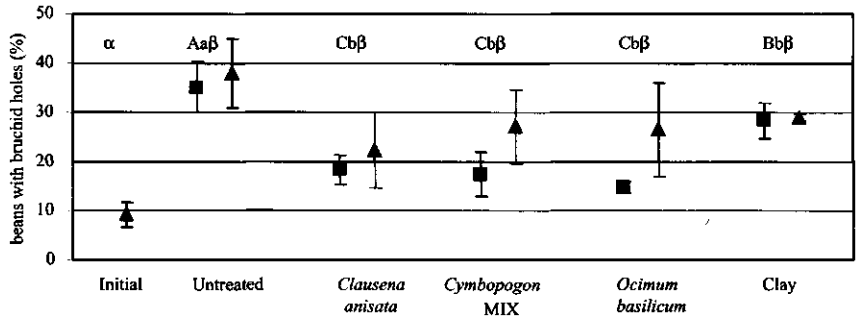
a


beans carrying bruchid eggs (%)
70
65
60
55
50
45
40
0
α
ABaα
Aaα
ABaα
Baα
ABaα
Initial
Untreated
Clausena anisata
Cymbopogon MIX
Ocimum basilicum
Clay

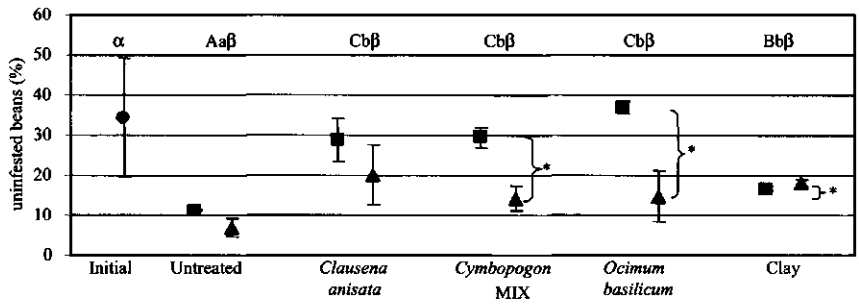
b


beans with signs of parasitism (%)
12
10
8
6
4
2
0
α
ABaβ
Abβ
ABaβ
ABaβ
Baβ
*
Initial
Untreated
Clausena anisata
Cymbopogon MIX
Ocimum basilicum
Clay

c


beans with bruchid holes (%)
50
40
30
20
10
0
α
Aaβ
Cbβ
Cbβ
Cbβ
Bbβ
Initial
Untreated
Clausena anisata
Cymbopogon MIX
Ocimum basilicum
Clay

d


uninfested beans (%)
60
50
40
30
20
10
0
α
Aaβ
Cbβ
Cbβ
Cbβ
Bbβ
*
*
*
Initial
Untreated
Clausena anisata
Cymbopogon MIX
Ocimum basilicum
Clay

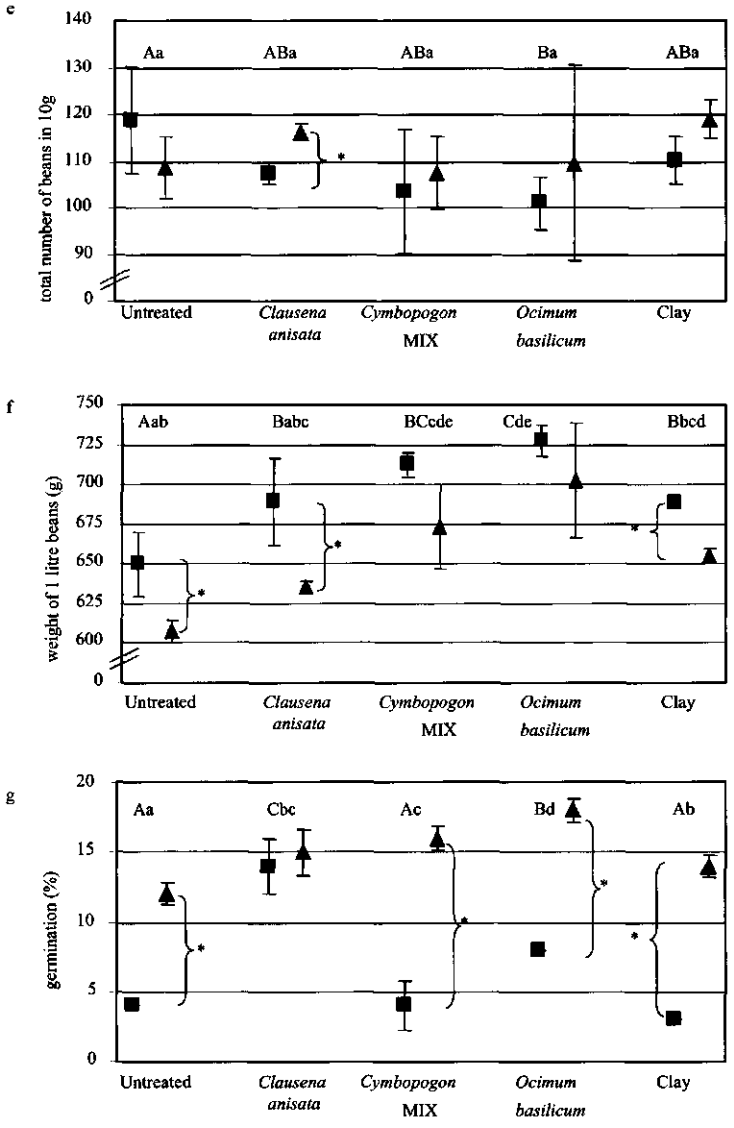


Figure 1 a - g: Differences in bruchid damage to beans after 236 days of storage without treatment, treated with finely ground clay or with volatile oils and clay in air-tight containers in Dannou (means ± sd, n = 3 for jerry-cans, n = 4 for canisters).
● = initial, ■ = jerry-cans, ▲= canisters. Among treatments, data points for jerry- cans accompanied by different capital letters showed differences ($P < 0.05$). For canisters, data points accompanied by a different lower case letter differ. Greek letters are used to indicate differences of the data after storage, irrespective of the container type, from the initially measured values. Within treatments, differences for the two container types are marked by a *.

in the clay treated control jerry-can samples. There was no difference within treatments between the container types.

The percentage of uninfested beans was lower after storage than at the beginning of the storage period for all containers. For all containers with oil treated beans and for the clay treated control, the percentage of uninfested beans was higher than in the untreated control. For all jerry-cans, there was a higher percentage of uninfested beans in the oil treated containers than in the clay treated control container. For all treatments except for *Clausena anisata*, the percentage of uninfested beans was higher in the jerry-cans than in the canisters. The highest percentage of uninfested beans was found in the jerry-cans treated with *Ocimum basilicum*.

The total number of beans per 10 g sample as a measure for bean damage, was equal for all treatments except for the *Ocimum basilicum* treated sample from the jerry-cans which contained fewer beans than the untreated jerry-can samples. For *Clausena anisata* treated beans, there were fewer beans in the samples from the jerry-cans than in those from the canisters.

The results from the measurements of the weight of one litre of beans were consistent with the seed counts on 10-g samples. For beans stored in jerry-cans, the weight of one litre untreated beans was lower than for any of the treatments. Beans treated with *Ocimum basilicum* were heavier than those treated with *Clausena anisata* or clay. The weight of one litre of beans was higher in jerry-cans treated with *Clausena anisata*, clay or untreated than for the comparable canisters. For the canisters, beans treated with *Cymbopogon* MIX and with *Ocimum basilicum* were heavier per litre than untreated control beans.

The percentage germination for *Clausena anisata* and *Ocimum basilicum* treated beans from jerry-cans was higher than for the beans from jerry-cans treated with *Cymbopogon* MIX and both controls. For the canisters, all treatments showed a higher percentage of germination than the untreated beans. For the controls and all treatments except *Clausena anisata*, the germination was higher for beans stored in canisters than for the respective jerry-cans.

After storage, the water absorbing ability of the beans was not affected by any of the treatments (data not shown). The moisture content of the beans was 12-15 % after storage, whereas it was 10.9 % before. The values for treated beans were never different from the untreated control (data not shown).

Generally, the jerry-cans are better storage containers than the canisters. All parameters evaluated indicated better protection except the percentage of parasitisation for which no differences were found and the germination where the results for the canisters were better.

Storage as pods with plant powders, Igana

When analysed in an ANOVA, the initial pod samples did not differ for any of the parameters measured. Therefore, the data of these measurements were all pooled and compared to the data collected after storage for treated and untreated granaries (Table 1).

The weight of 40 randomly picked pods treated with *Ficus exasperata* was not different after storage compared to the initial weight. For the pods treated with *Momordica charantia*, the weight had decreased but remained higher than that of the untreated pods. The pattern was the same for the weight of the seeds collected from these pod samples. The relative weight of the seeds in the pod was not different before or after storage for any of the treatments.

The weight of the seeds obtained from one kg pods after storage was higher for beans treated with *Ficus exasperata* than for the untreated or *Momordica charantia* treated beans. This was in accordance with the percentage of infested seeds, which was lowest for *Ficus exasperata* treated beans.

The numbers of bruchid beetles present in the samples of 40 pods after storage were not significantly different from the initial values. There were more beetles present in the untreated pods than in pods treated with *Ficus exasperata*.

There were more larval parasitoids after storage in the *Ficus exasperata* treated beans than for the other treatments and than initially measured.

Table 1: Evaluation of damage on cowpea stored in the pod. Initial damage and after 156 days of storage as affected by powders of *Ficus exasperata* and *Momordica charantia*. Data represent means of four (or nine for the initial data) measurements ± S.D. Values in rows followed by the same letter are not different (P > 0.05)

Parameter	Initial	Control	*Ficus exasperata*	*Momordica charantia*
Weight of 40 pods (g)	69.8 ± 8.8 a	40.4 ± 4.9 c	62.9 ± 5.3 ab	60.4 ± 1.5 b
Weight seeds from 40 pods (g)	57.5 ± 5.0 a	30.4 ± 3.9 c	50.2 ± 5.8 ab	45.5 ± 11.6 b
Seed weight as % of pod weight	73.9 ± 1.8 a	76.2 ± 14.2 a	79.7 ± 4.4 a	75.2 ± 18.6 a
Infested seeds (%)	[1]13.2 ± 2.8	[2]88.1 ± 4.2 a	[2]43.8 ± 8.0 b	[2]91.3 ± 6.4 a
Seeds parasitised by wasps (%)	4.9 ± 1.6			
1 kg pod seed weight (g)		408.9 ± 6.1 a	534.4 ± 28.3 b	415.8 ± 55.6 a
Number of beetles	12.3 ± 5.5 ab	15.8 ± 5.9 a	6.8 ± 3.0 b	13.8 ± 8.8 ab
Number of larval parasitoids	3.4 ± 2.4 a	1.5 ± 1.3 a	7.3 ± 2.2 b	2.5 ± 2.4 a

1: as measured on the total number of seeds

2: as measured on the total weight of seeds

Laboratory assays with oils

In the laboratory, comparing untreated and clay-treated control beans with beans treated with volatile oils, there were only few differences in effects on *Callosobruchus maculatus* caused by the oils (Table 2). The adult beetles, both males and female, on clay and oil treated beans died after fewer days than those on the untreated beans. The numbers of eggs after 24 h and laid during the whole life of the female beetle were not different. The percentage of pre-imaginal mortality was higher on untreated beans than on beans treated with clay alone or in combination with oils of *Clausena anisata* or *Ocimum basilicum*. The number of emerging beetles did not change due to the treatments.

Table 2: Effect of treatment of cowpea beans with ground clay and volatile plant oils on developmental parameters of *Callosobruchus maculatus* in a laboratory set-up (means of five ± S.D. Values in columns followed by the same letter are not significantly different ($P > 0.05$).

	Number of eggs		Longevity		Percentage	Number
	Day1	Total	Female	Males	mortality	emerged
Untreated control	15.4 ± 9.5 a	94.5 ± 18.5 a	8.5 ± 1.6 a	8.6 ± 1.3 a	26.5 ± 9.2 a	69.6 ± 16.3 a
Clay treated control	17.6 ± 9.5 a	88.8 ± 10.1 a	6.0 ± 0.9 b	4.7 ± 0.6 b	19.9 ± 7.8 b	71.3 ± 12.2 a
Clausena anisata	14.5 ± 7.3 a	90.4 ± 12.5 a	6.4 ± 0.6 b	5.3 ± 0.3 b	17.7 ± 12.4 b	74.8 ± 17.8 a
Cymbopogon MIX	14.2 ± 7.3 a	81.4 ± 20.3 a	5.8 ± 1.5 b	4.1 ± 1.0 b	22.6 ± 5.7 ab	63.4 ± 18.6 a
Ocimum basilicum	15.7 ± 6.7 a	80.8 ± 14.5 a	5.6 ± 0.9 b	5.0 ± 0.8 b	11.7 ± 4.4 b	71.6 ± 15.3 a

Effect on bean germination

Immediately after treatment, ethanol alone caused a 10% reduction of germination compared to untreated beans (Figure 2). Beans treated with oils of *Cymbopogon* MIX or *Ocimum basilicum* showed a lower germination than untreated beans. When the germination was tested two weeks after treatment, there was a negative effect of the treatment with ethanol, but the oils did not decrease the percentage of germinated beans compared to the ethanol treatment. When compared to untreated beans, all treatments had a negative effect on germination. Within the treatments, the effect on germination was not influenced by the time interval between treatment and measurement.

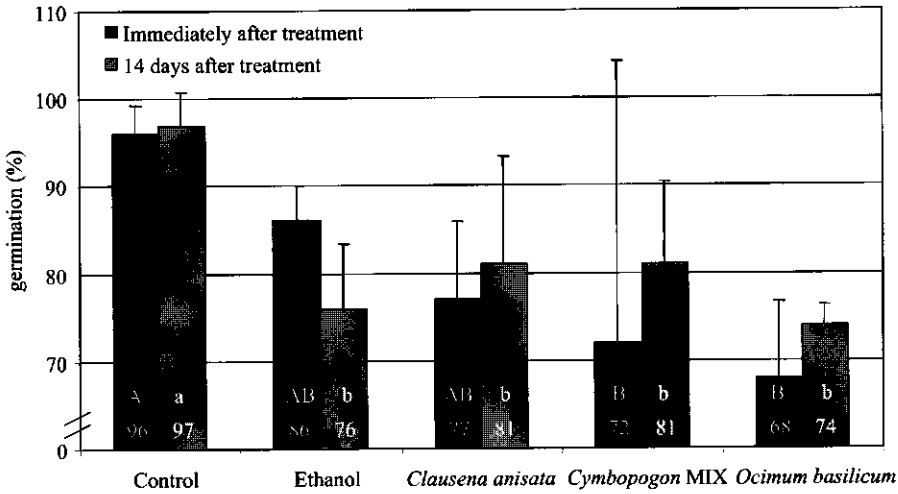


Figure 2: Percentage of germinated cowpea beans (means of 4*25 seeds ± S.D.) after treatment with oils. Bars with the same letter are not significantly different ($\alpha = 0.05$). Capital letters are used for the test immediately after treatment, small letters for the test two weeks after treatment.

Discussion

Treatment of beans with plant products can be effective to reduce the damage done by seed beetles. After storage, the percentage of infested beans was higher than before for both storage as pods and as loose grains. However, when compared to the untreated samples, the oils reduced the number of beetles emerging from the beans while the number of uninfested beans, the weight of the beans and the germination were higher. The plant powders as treatments for pods reduced the loss of seed weight in the pods and *Ficus exasperata* reduced the percentage of infested seeds considerably. Moreover, there were more parasitoids present in the pods treated with *Ficus exasperata,* which could point to a selective negative effect on the beetle but not on its major natural enemies.

The use of plant powders as insecticides on stored products has been reported frequently (Part I of this thesis), but on the powders we used not much information exists. The leaf powder of *Ficus exasperata* did not affect oviposition or hatching for *C. maculatus* (Ofuya, 1990) and it did not show toxic or repellent effects on this beetle (Chapter 3). Topical application of an ethanol extract of leaves of *Ficus perforata* caused elevated mortality rates for adults of another storage pest, the confused flower beetle *Tribolium confusum* (Williams & Mansingh, 1993). The traditional use of species of *Ficus* (i.e. *F. gnaphalocarpa* and *F.*

sycomorus) as insecticides for seed storage has been reported from Niger, but their efficacy is not known (Anonymous, 1984; Hamidou, 1996; Maiga, 1989). Leaves of *Ficus exasperata* are used as traditional medicines against ulcers or stomach disorders (Akah *et al.*, 1998).

In the laboratory, leaf powder of *Momordica charantia* was repellent to *C. maculatus*, but in a no-choice situation it did not have any effect on the development of this beetle (Chapter 3). The plant contains momordicines which have anti-feedant effects even to insects specialised on the plant family of Cucurbitaceae (Abe & Matsuda, 2000). Acetone extracts of the leaves were highly toxic to cowpea aphids (Ofuya & Okuku, 1994) and to their predators, coccinellid beetles (Ofuya, 1997b). More effects of this plant species on stored products-infesting beetles are not documented.

Volatile oils are here presented as insecticides, but they are also used in aroma therapy, in perfumes, pharmaceuticals and as food flavouring. Of the more than 3000 essential oils known today (Anonymous, 2000), three were selected because the plants they were extracted from (except *Cymbopogon flexuosus*, an East-Asian species), occur in Benin and the oil is easily obtained and has a relatively high yield. All oils tested here had a repellent effect on *Callosobruchus maculatus* in the laboratory, but no toxicity to the beetle in a no-choice situation was demonstrated (Chapter 4).

The oil of *Clausena anisata* is used as an insect repellent, but also as a medicinal flavour (Axtell & Fairman, 1992). Throughout Africa, the plant is used as a heart tonic, anthelminthic, parasiticide, purgative and for the treatment of rheumatism, malaria, influenza and other ailments (Gebreyesus & Chapya, 1983). The oil has anti-bacterial and anti-fungal properties (Gundidza *et al.*, 1994). The composition of the oil varies with the origin of the plant. Oil extracted in Benin yielded mainly methyl-chavicol, limonene and (*E*)-anethole (Ayedoun *et al.*, 1997). The anethole in the oil was reported to have toxic and anti-fertility effects on the Mediterranean fruit fly *Ceratitis capitata* (Bazzoni *et al.*, 1997). The effect on stored product beetles is not known.

The effect of the mixture of oils of *Cymbopogon citratus* and *Cymbopogon flexuosus* on *Callosobruchus maculatus* as we used in this study, has never been reported. Of the insecticidal effect of the latter species, no records exist. *Cymbopogon citratus* was effective against *Callosobruchus maculatus* as an oviposition inhibitor for up to 90 days after treatment (Bhaduri *et al.*, 1990) and it could protect stored cowpea by its fumigant effect (Gbolade & Adebayo, 1993). The eggs that were laid did not hatch and the larvae could not penetrate the bean (Ketoh *et al.*, 1998). The oil had a pronounced fungitoxic effect (Mishra & Dubey, 1994) as well.

The oil of *Ocimum basilicum* is traditionally used in West Africa as an insecticide. The oil contains estragole, cineole, pinene, alkaloids, glucosides, saponine acid, sterol and triterpenes (Dabire, 1991) or linalool, eugenol, trans-bergamotene, terpinen-4-ol, T-cadinol and 30 other compounds in smaller quantities (Djibo *et al.*, 1996). It was toxic to

Callosobruchus maculatus adults upon fumigation (Kéïta *et al.*, 2000). The oil had an adverse effect on bruchid emergence through early larval mortality and it was repellent to adult beetles (Papachristos & Stamopoulos, 2002). The oil, apart from insect repellent activity, also had anti-fungal activity (Dube *et al.*, 1989; Montes-Belmont & Carvajal, 1998).

The anti-bacterial and especially the anti-fungal effects that are found for the oils, are important advantages of these insecticides. Beetle infestation in beans causes an increase of the incidence of moulds such as species of *Alternaria*, *Aspergillus*, *Curvularia*, *Fusarium*, *Penicillium* and *Rhizopus* (Charjan & Tarar, 1994) which could be prevented or decreased with the plant oils.

Beans stored in jerry-cans were generally preserved better than in canisters. All parameters, except the percentage of parasitisation and the germination were at least slightly better for the beans stored in jerry-cans. In canisters, the lid was transparent whereas in jerry-cans no light could reach the stored product. Moreover, the jerry-cans were probably better air-tight than the canisters. The lack of oxygen could have had a negative effect on the developing beetles and parasitic wasps (Caswell, 1973; Singh & Yadav, 1991).

For the data collected in Igana, there is a large unexplained difference between the relative weight of the seeds collected from one kg pods, which is about 45 percent, and the relative weight of seeds collected from 40 randomly selected pods, which is about 77 percent.

The intact pod protects the beans from bruchid attack to a certain extent (Kitch *et al.*, 1991; Kitch & Shade, 1993; Ofuya & Awelewa, 1993). In many of the seeds we collected from Igana, we found various developmental stages of beetles that had failed to develop completely (unpublished results).

The results we found in the laboratory tests presented here cannot fully explain our findings from the field test. In the laboratory, the treatment of beans with clay alone had as much effect on the reproducing and developing beetles as treatment of the beans with volatile oils mixed with clay. When comparing figures 1a and c with the data presented in table 2, the data for the canisters are similar to those found in the laboratory, but the data for the jerry-cans show differences. The differences might have been caused by the petri dishes we used. These dishes are much smaller than the containers used in the field experiment and the beetles move through the whole petri dish. Thus, the beetles in the petri dishes come more into contact with the abrasive clay that inevitably assembles partly at the bottom of the container. Moreover, we used petri dishes with ridges on the lid, which made them less air-tight than the jerry-cans. Therefore, the effect of the clay might be overestimated in the laboratory set-up compared to the effect of the oil. In the jerry- cans, all oils performed better than the clay treatment for the percentage of seeds with bruchid holes and for the percentage of uninfested seeds, and *Ocimum basilicum* oil had a better effect on the weight of one litre of beans and on the germination of the stored seeds. Non-volatile oils like that of neem (*Azadirachta indica*) have been reported to reduce seed germination (Gupta *et al.*,

1988) whereas for volatile oils, no such effect has been reported (Part I of this thesis). Germination is also affected by bruchid damage. The number of emergence holes in a bean is directly proportional to a reduction in germination (Baier and Webster, 1992).
An important difference between most laboratory tests and field tests is that the former usually report the preventive effect. In most laboratory tests, uninfested seeds are used, whereas in field tests one cannot prevent starting with infested material. Material from the field is likely to be infested with bruchid eggs already at the time of harvest (Van Huis, 1991; Prevett, 1961). To keep the stored product as clean as possible, the treatment with plant material should therefore be effective at least against the first generation of emerging and reproducing insects and reinfestation should be prevented.
In this study, the local traditional practices were taken into account as much as possible. All tests were done in cooperation with the farmers and if possible, they were involved in the actual treatment. The cowpeas were of the locally preferred and most cultivated varieties and the locally commonly used storage practices were applied. The purpose of this approach was to make the threshold for adoption of the methods, if they proved effective, as low as possible. Moreover, in this way, the farmers could immediately see the effects of easily obtained plant products on the storability of their products and compare the results to earlier seasons when the beans had been left untreated.
Such a participatory approach had the disadvantage that the experimental set-up we used was dependent on the availability and the quantity of freshly harvested, untreated cowpea of a certain variety. If more beans and more storage facilities had been present in the villages, the testing could have been more extensive. However, even from the results presented here, the effect of plant materials as insecticides in traditional storage is obvious. The plants, especially the leaf powder of *Ficus exasperata* and the volatile oil of *Ocimum basilicum* help to keep the bruchid population within acceptable numbers.

Acknowledgements

The research was financed by the Centre Béninois pour le Développement Durable. We would like to thank the farmers for their patience, their faith in our methods and for their cooperation. We are grateful to D. Biaou and the project partners in Dannou and Igana for the organisation and facilities. Special thanks are expressed to L. Saka and H. Kassa for their help in the laboratory. The data transportation by Y. Jongema was highly appreciated.

Chapter 8

Summary and conclusions

Plants as producers of secondary metabolites could be used to protect other plants and their products from insect damage. Especially if other means of protection are not available, such plants that negatively interfere with insect behaviour and reproduction could be useful in crop protection. This thesis focuses on the protection of stored cowpea beans (*Vigna unguiculata*), against the attack of the cowpea beetle *Callosobruchus maculatus* with plant products.

Cowpea grows in all tropical areas, and in West Africa it is one of the more important crops for resource-poor farmers. The beans are rich in protein and are therefore known as "the meat of the poor". The cowpea beetle is present wherever there is cowpea and it needs only ripe cowpea beans for its reproduction. The beetle lays its eggs on the seeds and the development of the larva and pupa takes place inside the bean from which the adult beetle emerges. Therefore, this beetle does very well in storage rooms where the seeds are kept until they are needed for consumption, trade or sowing. Beans that are severely attacked by beetles are not suitable for human consumption, are worthless on the market and will not germinate. In West Africa, the means for protection of crops against insects are often limited and the cowpea growers have few possibilities to treat their harvested beans to prevent insect damage. This thesis presents the search for cheap, safe and available means with and for farmers in Benin in West Africa to prevent or minimise the reproduction of the cowpea beetle in store. The approach that was used to tackle the problem is depicted in Figure 1.

In a review of the literature on botanical protectants of stored seeds against beetle damage (part I of this thesis), plant products were listed and discussed for the effects and the specific advantages and disadvantages when powders, ash, volatile or non-volatile oils or extracts of the plants were used. It appeared that no standardised test procedures have been developed so far and no ranking could be given for the efficacy of the plant species. The plants originated from all over the world and the tests that had been performed to investigate their effects on beetles as well as the results of these varied for all parameters.

The second part of the thesis focussed on the protection of stored cowpea against its specialised seed beetle with plants that are traditionally used for such a purpose in Benin. The reproductive success of the cowpea beetle may vary with the origin of the beetle and between varieties of the host seeds. Studies on the efficacy of preparations harmful to insects are best done on a very successful beetle strain on a very susceptible bean variety. To find the most suitable beetles of Benin and the best bean variety available, in chapter 2, life history parameters of beetle strains from three different origins in West Africa were compared on two susceptible varieties of cowpea. All beetle strains were tested in a no-

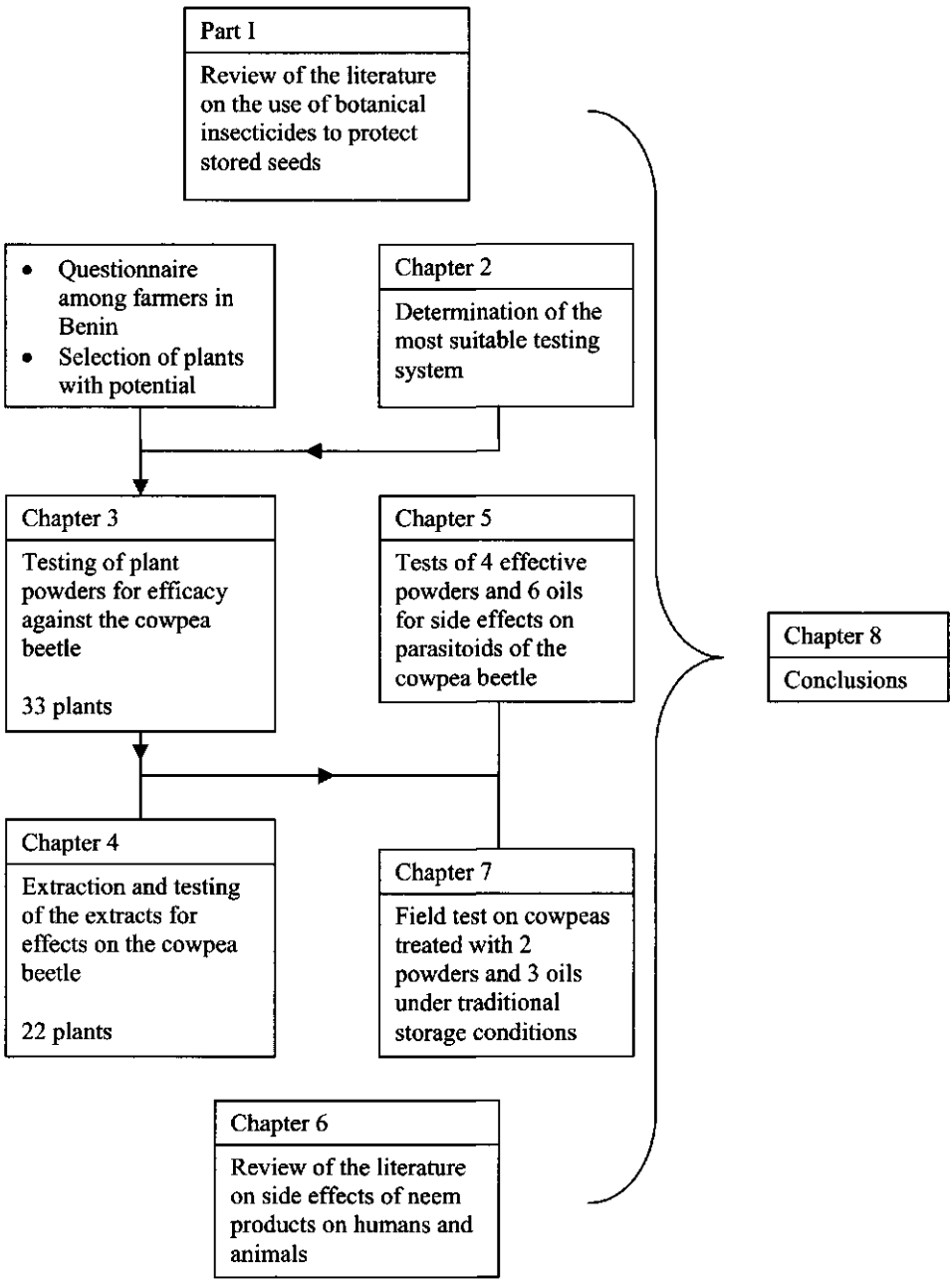


Figure 1: Scheme according to which the research question of this thesis was tackled

choice and a two-choice situation. No major differences were found between the beetle strains. The developmental period from egg to adult was prolonged on the bean variety Kpodjiguegue. In a two-choice situation, the beetles showed a strong preference for the Californian blackeyed bean variety to oviposit on. For further research, a beetle strain from Benin was used on the highly preferred Californian blackeyed beans. This combination was subsequently used in all laboratory experiments.

On this testing system, the effect of plants as a means to repel the beetle or to interfere with its' reproduction was investigated. First, a questionnaire was held among farmers in Benin to find out which plant species they used traditionally for the protection of stored cowpea. The plants mentioned most often were collected and the powders of dried material of 33 plant samples were tested in the laboratory for their toxic and repellent effects against the cowpea beetle (chapter 3, Table 1 summarises the findings). Effects on development and reproduction were evaluated measuring life history parameters in a no-choice situation. Leaf powders of tobacco (*Nicotiana tabacum*), tephrosia (*Tephrosia vogelii*) and the violet tree (*Securidaca longepedunculata*) reduced the number of emerging beetles. Repellence was evaluated observing female beetles exposed to treated and untreated beans in a linear olfactometer. Leaf powders of clausena (*Clausena anisata*), dragontree (*Dracaena arborea*), tephrosia, bittergourd (*Momordica charantia*) and false tobacco (*Blumea aurita*) were most repellent to the beetle.

To investigate if the insect repellent or toxic effects of these plants could be enhanced, the plants that were effective as powders were extracted with hot water (chapter 4). The simple extraction equipment with water as a solvent was chosen so that it could be easily used at larger scale and applied with little investment in Africa as well. Extractions resulted in thirteen volatile oils, two non-volatile oils and eight slurries. These were all tested for their toxic and repellent effects. Application of volatile oils led in most cases to a reduced number of eggs on treated beans. The volatile oils of citronella grass (*Cymbopogon nardus*) and camel grass (*C. schoenanthus*) caused the majority of the eggs not to develop into adult beetles. Repellent effects were found for volatile oils of clausena, lemon grass (*C. citratus*), citronella grass, a mixture of lemon grass and east Indian lemon grass (*C. flexuosus*), marubio (*Hyptis spicigera*), marigold (*Tagetes minuta*) and for two different samples of basil (*Ocimum basilicum*). Non-volatile oils were not repellent and had no effect on the number of eggs laid, but the development of these eggs was hampered, most so by neem (*Azadirachta indica*) oil. None of the slurries had a toxic effect on the beetles, but the slurries obtained from papaya (*Carica papaya*), dragontree and tephrosia were repellent. Oils, both volatile and non-volatile were easily extracted from plant material and showed promising results as a protective agent for stored cowpea. This simple extraction did not always enhance or retain the efficacy of a plant powder, but when an oil could be extracted, the efficacy of the plant was enhanced. The mechanisms of action of volatile and non-

Table 1: Results of laboratory and field tests with traditionally used plant products for the protection of stored cowpea against the cowpea beetle

Plant species	Common name	Powder	Extraction	Extract	Parasitoids	Field
Annona muricata	Soursop	--	*	*	*	*
An. senegalensis a[1]	Custard apple	TA	*	*	*	*
An. senegalensis b[1]	Custard apple	R	*	*	*	*
Azadirachta india	Neem oil	*	Oil	T	E	*
Az. Indica a[1]	Neem	A	*	*	*	*
Az. Indica b[1]	Neem	*	*	*	El	*
Az. Indica t[1]	Neem	A	*	*	*	*
Az. Indica z[1]	Neem	--	Slurry	A	*	*
Blumea aurita	False tobacco	R	Oil	A	El	*
Capsicum frutescens	Pepper	A	Slurry	--	*	*
Carica papaya	Papaya	R	Slurry	R	*	*
Chamaecrista nigricans	Moutounditimou	A	*	*	*	*
Clausena anisata a[1]	Clausena	TR	Oil	--	*	*
Cl. anisata b[1]	Clausena	*	Oil	R	--	F
Combretum micranthum	Combretum	R	Slurry	--	*	*
Crateva religiosa	Boumbari	A	*	*	*	*
Cymbopogon citratus	Lemon grass	--	Oil	TR	*	*
Cy. MIX		*	Oil	R	E	F
Cy. nardus	Citronella grass	*	Oil	TR	E	*
Cy. schoenanthus	Camel grass	*	Oil	T	--	*
Dracaena arborea a[1]	Dragontree	R	*	*	*	*
D. arborea m[1]	Dragontree	R	Slurry	R	*	*
Ficus exasperata	Ficus	A	*	*	*	F
Helianthus annuus	Sunflower	*	Oil	T	*	*
Heliotropium indicum	Heliotrope	R	*	*	*	*
Hyptis spicigera a[1]	Marubio	A	Oil	R	*	*
Hy. spicigera b[1]	Marubio	--	*	*	*	*
Hy. suaveolens	Pignut	TA	*	*	*	*
Iboza multiflora	Iboza	R	*	*	*	*
Khaya senegalensis	Mahogany	--	*	*	*	*
Momordica charantia	Bittergourd	R	Slurry	--	*	F
Moringa oleifera	Horseradish tree	T	*	*	*	*
Nicotiana tabacum	Tobacco	TR	Oil	--	EL	*
Ocimum basilicum b[1]	Sweet basil	--	Oil	R	--	F

Plant species	Common name	Powder	Extraction	Extract	Parasitoids	Field
Oc. Basilicum t[1]	Sweet basil	*	Oil	TR	*	*
Opilia celtidifolia	Opilia	--	*	*	*	*
Pergularia daemia	Pergularia	--	*	*	*	*
Securidaca longepedunculata a[1]	Violettree	A	*	*	*	*
S. longepedunculata b[1]	Violettree	TA	Slurry	--	*	*
Tagetes minuta	Marigold	--	Oil	R	*	*
Tephrosia vogelii	Tephrosia	TR	Slurry	R	EL	*

1: abbreviation of the region of origin of the plant, see chapter 3 & 4

-- = no effect found

* = not investigated

A = attractive

E = harmful to the beetle's egg parasitoid

F = tested and found effective in the field test

L = harmful to the beetle's larval parasitoid, l = not harmful to the larval parasitoid

R = repellent

T = toxic: negative effect on reproduction

volatile oils are different, but both seemed promising as means to prevent beetle damage. Thus, quite a few plants were effective as powders or as extracts. However, until now only the effect of such botanicals on the pest insect had been investigated. The indirect effect of the products on the storability of seeds through the impact on the natural enemies of the pest was investigated as well (chapter 5). Four plant powders and six plant oils with a known effect on the cowpea beetle were tested for their possible negative side effects on the beetles' egg parasitoid *Uscana lariophaga* and the larval parasitoid *Dinarmus basalis*. All treatments caused a reduction in parasitisation by the egg parasitoid and powders of tobacco and tephrosia negatively affected its development. In a two-choice situation, in a linear olfactometer, the egg parasitoid was repelled by most of the oils. Oviposition by the larval parasitoid was hampered after treatment of the beans with plant powders, but the eggs that were laid developed normally. In a Y-tube olfactometer, this larval parasitoid did not discriminate between odours of untreated and plant-powder-treated beans. The powders of tobacco and tephrosia had stronger negative effects on the two parasitoids than the powders of neem and false tobacco. In untreated samples collected from traditional storage facilities and treated with plant powders in the laboratory, none of the treatments could prevent the build-up of a beetle population. At 24 days after treatment, the largest numbers of beetles had emerged from beans treated with powders of tobacco and tephrosia, possibly due to the side effects on the parasitoids. The botanicals tested here negatively affected parasitoids, but

powders of neem and false tobacco may be compatible with biological control by the larval parasitoid since they negatively affect the beetle, but do not have severe negative effects on this parasitoid.

Other possible side-effects of insecticides were also investigated: those on the human consumers of the beans. The fact that the products are used traditionally could imply that they are reasonably safe, or that at least treatment with these products causes less harm than the hunger would if the beans were not treated. However, since the plant products are all complex and quite variable mixtures of many compounds, a detailed description of their toxicity would require extensive studies. To evaluate possible side effects of botanical insecticides on humans, the available literature was consulted for the most famous source of botanical insecticides: the neem tree (chapter 6). A study was done on the toxicological data from human and animal experiments with oral administration of different neem-based preparations. Beneficial effects, such as blood sugar lowering properties, anti-parasitic, anti-inflammatory, anti-ulcer and hepatoprotective effects were often reported, but toxic effects were also found. For all preparations, reversible effects on reproduction of both male and female mammals are reported. From the available data, safe doses were calculated that were compared to the ingestion of residues on beans treated with neem preparations. Newly collected parts of the neem tree should be used for the preparation, which are free from fungal infestation and thus not contaminated with aflatoxins. If the residues of the insecticide are thoroughly washed off the treated beans before cooking, the use of neem as an insecticide would be safe.

All work reported up till now was done in the laboratory, but the effects of the botanical insecticides might be different when they are applied in traditional storage devices. Therefore, the effect of plant products on the cowpea beetle were investigated in a field set-up (chapter 7). Seeds, mixed with finely ground clay and three volatile oils were stored in air-tight jerry-cans and canisters, and pods were treated with leaf powders of two plant species and stored in traditional palm-leaf huts. Beetle damage was evaluated before and after storage. The applied treatments could not prevent damage, but after treatment with oils, fewer beans showed beetle emergence holes and the percentage of uninfested beans and the weight of one litre of beans were higher. Germination of stored beans was best after treatment with basil oil. Leaf powder of bittergourd was effective against weight loss of stored pods, whereas ficus (*Ficus exasperata*) also caused a decreased percentage of infested beans, a lower number of emerged beetles and more parasitoids emerged. Laboratory tests on the effect of oils on the development of the beetle and on bean germination did not reveal effects of the treatments. For the non-volatile oils and the slurries, no tests were done in the field.

When the beans are to be protected from the cowpea beetle in an integrated pest management (IPM) approach, the disadvantageous effect of the plant products on parasitoids

should be outweighed against their inhibiting effect on the beetle. It is promising that in the field test signs of parasitisation were found in all samples.

With the help of farmers and their age-old practices, traditional methods for the protection have been put to the test. In the search for botanical protectants, among the traditionally used plants, examples were found that are effective (see table 1). The fact that they did not all show effects in our tests could be due to the dose used being too low or to the fact that the plant material was at least a few months old before it was used in our laboratory tests. It could also be that the particular sample of a plant, grown under other conditions might have had an effect. This was shown for some plants where several samples from different origins were tested. The results of these tests always differed among these samples. Anyhow, important differences in protective efficacy were found between traditionally used plant products.

Moreover, as shown from the field test, the results from the laboratory cannot always predict what will happen in the field. The sample of ficus tested in the laboratory, did not show any effect against the beetle, whereas in the field test, this plant was more effective than bittergourd which in the laboratory was repellent to the beetle. This effect of bittergourd in a two-choice situation in the laboratory was obvious, but in the field, the effect was not so pronounced that the beetles would leave the storage room even though they could easily do so. If there had been an untreated bean stock nearby, the effect might have been more obvious.

The plant products that were tested in the laboratory on uninfested beans could only in a few cases prevent infestation and when tested on beans that were already infested the products did not completely stop the growth of the beetle population. In many cases, the beetle population grew more slowly on treated beans, resulting in fewer beans being infested. On the market in Benin, people were observed to deliberately buy beans that were slightly infested with insects to be certain that the beans had not been treated with noxious chemicals (pers. observation). Treatment with plant products will probably give such result, not many emergence holes and few insects present in the store house.

From the results presented in this thesis, it can be concluded that the use of selected plant materials, especially of volatile oils for the protection of stored cowpea in Benin seems feasible. Toxicity, residual effects, persistence and field effects, especially of slurries are still to be investigated more profoundly. The methods could be applicable for other stored seeds as well.

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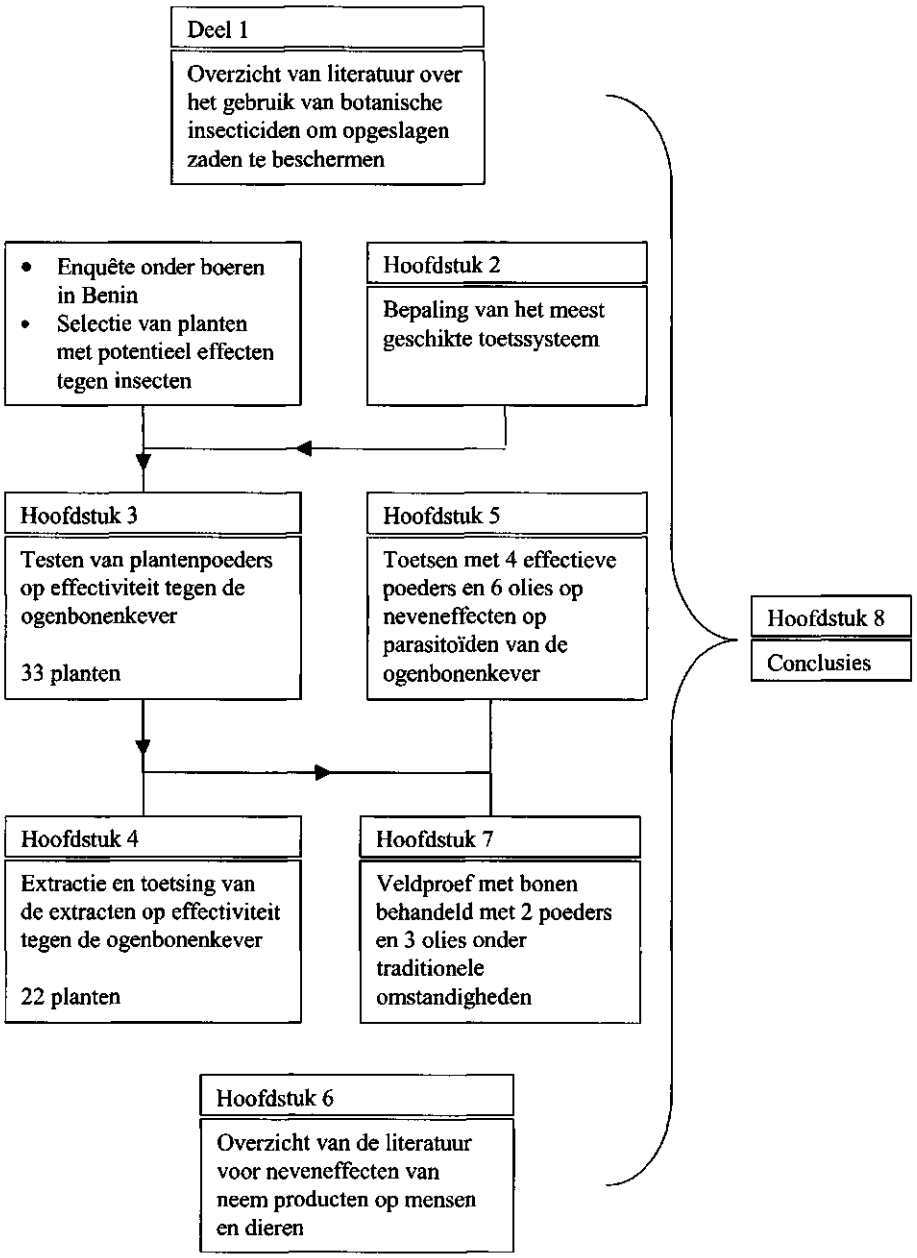
Samenvatting en conclusies

Planten, de producenten van secundaire metabolieten kunnen gebruikt worden om andere planten en hun producten te beschermen tegen schade door insecten. Zeker als andere beschermingsmiddelen niet beschikbaar zijn, kunnen planten, die het gedrag of de voortplanting van insecten negatief beïnvloeden, nuttig zijn in de gewasbescherming. Dit proefschrift gaat in op de bescherming van opgeslagen ogenbonen (*Vigna unguiculata*) tegen de schade door de ogenbonenkever *Callosobruchus maculatus* door middel van plantenproducten.

De ogenboon groeit in alle tropische gebieden en in West Afrika is het een belangrijk gewas met name voor arme boeren. De bonen hebben een hoog eiwitgehalte en staan daarom bekend als "het vlees van de armen". De ogenbonenkever is overal aanwezig waar de bonen zijn en hij heeft genoeg aan de rijpe zaden voor zijn voortplanting. De kever legt de eieren op het zaad en de ontwikkeling van de larve en de pop voltrekt zich binnenin de boon waar dan een volwassen kever uit tevoorschijn komt. Deze kever floreert in opslagruimten waar de zaden worden bewaard tot ze nodig zijn voor consumptie, handel of als zaaigoed. Bonen die ernstig zijn aangetast door de kever zijn niet meer geschikt voor menselijke consumptie, zijn waardeloos voor de verkoop en kiemen niet meer. In West Afrika zijn de middelen om gewassen te beschermen tegen insecten vaak schaars en de verbouwers van ogenbonen hebben slechts weinig mogelijkheden om hun geoogste bonen te behandelen om schade door insecten te voorkomen. In dit proefschrift wordt verslag gedaan van de zoektocht naar een goedkoop, veilig en beschikbaar middel met en voor boeren in Benin in West Afrika om de voortplanting van de ogenbonenkever in de bonenopslag te voorkomen of in ieder geval te minimaliseren. De benadering die gebruikt is om deze zoektocht tot een goed einde te brengen is weergegeven in figuur 1.

In een overzicht van de literatuur over botanische beschermingsmiddelen voor opgeslagen zaden tegen schade door kevers (deel I van dit proefschrift), wordt een lijst gegeven van de gebruikte plantenproducten en worden de specifieke voor-, en nadelen bediscussieerd wanneer die planten als poeder, as, vluchtige of niet-vluchtige olie of als extract worden toegepast. Het blijkt dat er tot nu toe geen gestandaardiseerde testprocedures zijn en dat er geen rangorde gegeven kan worden voor de effectiviteit van de plantensoorten. De planten waarover is gerapporteerd en om hun effectiviteit tegen kevers te toetsen zijn niet gestandaardiseerd.

Het experimentele werk (dit deel II van het proefschrift) richtte zich op de bescherming van opgeslagen ogenbonen tegen de gespecialiseerde kever gebruik makend van planten die van oudsher voor dat doel toegepast werden in Benin. Het voortplantingssucces van de ogenbonenkever kan variëren met zijn oorsprong en met de variëteit van het gastheerzaad. Studies naar de effectiviteit van preparaten die schadelijk zijn voor de insecten kunnen het best gedaan worden met een keverstam met een grote reproductiecapaciteit op een zeer



Figuur 1: Schema van aanpak zoals het gebruikt is in dit proefschrift voor het beantwoorden van de onderzoeksvraag

vatbare bonenvariëteit. Om de meest geschikte keverstam en de best mogelijke twee-keuze situatie. De keverlijnen vertoonden onderling geen belangrijke verschillen in ontwikkeling. De ontwikkelingsperiode van ei tot volwassen kever was langer voor de kevers op de boonvariëteit Kpodjiguegue. In een twee-keuzeproef lieten de kevers een grote voorkeur zien voor de Californische zwartoogboon om eieren op te leggen. Voor de rest van de proeven in het laboratorium zijn altijd kevers uit Benin gebruikt op de geprefereerde Californische zwartoogboon.

Met dit toetssysteem werd het effect van planten onderzocht als middel om de kevers af te stoten of om de reproductie te reduceren. Om te beginnen was er een enquête gehouden onder de boeren in Benin om er achter te komen welke plantensoorten zij van oudsher gebruikten ter bescherming van hun opgeslagen ogenbonen. De meest genoemde planten werden verzameld en de poeders van droog materiaal van 33 plantenmonsters werden in het laboratorium getoetst op hun giftige en hun afstotende effect op de ogenbonenkever (hoofdstuk 3, in Tabel 1 zijn de resultaten samengevat). Effecten op de ontwikkeling en de voortplanting werden beoordeeld door voortplantingsparameters te meten in een geen-keuze experiment. Bladpoeders van *Nicotiana tabacum* (tabak), *Tephrosia vogelii* (tephrosia) en *Securidaca longepedunculata* verminderden het aantal uitgekomen volwassen kevers. Afstoting werd gemeten door een vrouwtjeskever te bekijken die werd blootgesteld aan behandelde bonen aan de ene, en onbehandelde bonen aan de andere kant van een glazen buis (lineaire olfactometer). Bladpoeders van *Clausena anisata* (clausena), *Dracaena arborea* (drakenboom), tephrosia, *Momordica charantia* (bittere kalebas) en *Blumea aurita* (valse tabak) waren het meest afstotend voor de kever.

Om vast te stellen of de insecten-afstotende of de giftige werking van de planten kon worden versterkt, werden die planten waarvan de poeders effectief waren, geëxtraheerd met heet water (hoofdstuk 4). Er werd gekozen voor een simpele extractie-installatie en voor water als oplosmiddel zodat de procedure eenvoudig op grotere schaal kan worden toegepast en men met weinig investeringen ook in Afrika de extractie zou kunnen uitvoeren. Er werden dertien vluchtige oliën gewonnen, acht slurries en twee niet-vluchtige oliën. Deze extracten werden weer getoetst op hun giftige en afstotende effecten. De toepassing van de vluchtige oliën leidde in de meeste gevallen tot een kleiner aantal kevereieren op de behandelde bonen. De vluchtige oliën van *Cymbopogon nardus* (citronellagras) en *C. schoenanthus* (kamelengras) verhinderden de ontwikkeling van het merendeel van de eieren tot volwassen kevers. Afstotende effecten werden gevonden voor de vluchtige oliën van clausena, *C. citratus* (citroengras), citronella gras, een mengsel van citroengras en *C. flexuosus* (Oost-Indisch citroengras), *Hyptis spicigera* (marubio), *Tagetes minuta* (Afrikaantje) en voor twee verschillende oliemonsters van *Ocimum basilicum* (basilicum). Niet-vluchtige oliën waren niet afstotend en hadden geen invloed op het aantal eieren dat gelegd werd, maar de ontwikkeling van de eieren werd verhinderd, het meest door *Azadirachta indica* (neem) olie. Geen van de slurries had een giftige werking op de kevers, maar de slurries verkregen na

Tabel 1: Resultaten van laboratorium en veldtoetsen met van oudsher gebruikte planten ter bescherming van opgeslagen bonen tegen de ogenbonenkever.

Plant	Poeder	Extractie	Extract	Parasitoïden	Veld
Annona muricata	--	*	*	*	*
Annona senegalensis a[1]	TA	*	*	*	*
Annona. senegalensis b[1]	B	*	*	*	*
Azadirachta indica	*	Olie	T	E	*
Azadirachta indica a[1]	A	*	*	*	*
Azadirachta indica b[1]	*	*	*	El	*
Azadirachta indica t[1]	A	*	*	*	*
Azadirachta indica z[1]	--	Slurry	A	*	*
Blumea aurita	B	Olie	A	El	*
Capsicum frutescens	A	Slurry	--	*	*
Carica papaya	B	Slurry	B	*	*
Chamaecrista nigricans	A	*	*	*	*
Clausena anisata a[1]	TB	Olie	--	*	*
Clausena anisata b[1]	*	Olie	B	--	F
Combretum micranthum	B	Slurry	--	*	*
Crateva religiosa	A	*	*	*	*
Cymbopogon citratus	--	Olie	TB	*	*
Cymbopogon MIX	*	Olie	B	E	F
Cymbopogon nardus	*	Olie	TB	E	*
Cymbopogon schoenanthus	*	Olie	T	--	*
Dracaena arborea a[1]	B	*	*	*	*
Dracaena arborea m[1]	B	Slurry	B	*	*
Ficus exasperata	A	*	*	*	F
Helianthus annuus	*	Olie	T	*	*
Heliotropium indicum	B	*	*	*	*
Hyptis spicigera a[1]	A	Olie	B	*	*
Hyptis spicigera b[1]	--	*	*	*	*
Hyptis suaveolens	TA	*	*	*	*
Iboza multiflora	B	*	*	*	*
Khaya senegalensis	--	*	*	*	*
Momordica charantia	B	Slurry	--	*	F
Moringa oleifera	T	*	*	*	*
Nicotiana tabacum	TB	Olie	--	EL	*
Ocimum basilicum b[1]	--	Olie	B	--	F
Ocimum Basilicum t[1]	*	Olie	TB	*	*
Opilia celtidifolia	--	*	*	*	*

Plant	Poeder	Extractie	Extract	Parasitoïden	Veld
Pergularia daemia	--	*	*	*	*
Securidaca longepedunculata a[1]	A	*	*	*	*
Securidaca longepedunculata b[1]	TA	Slurry	--	*	*
Tagetes minuta	--	Olie	B	*	*
Tephrosia vogelii	TB	Slurry	B	EL	*

1: afkorting van de regio van afkomst van de plant; zie hoofdstuk 3 & 4

-- = geen effect gevonden

* = niet onderzocht

A = aantrekkelijk voor de kever

B = afstotend voor de kever (Bah!)

E = schadelijk voor de eiparasitoïde van de kever

F = getoetst en effectief bevonden in de veldproef

L = schadelijk voor larvale parasitoïde van de kever, l = onschadelijk voor larvale parasitoïde

T = toxisch: negatief effect op de voortplanting van de kever

extractie van *Carica papaya* (papaja), drakenboom en tephrosia waren afstotend. De oliën, vluchtige en niet-vluchtige konden eenvoudig uit de planten geëxtraheerd worden en gaven veelbelovende resultaten als beschermingsmiddelen voor opgeslagen ogenbonen. De simpele extractiestap kon niet in alle gevallen de effectiviteit versterken of behouden, maar als er een olie gewonnen werd, was die effectiever dan de onbewerkte plant. De werkingsmechanismen van vluchtige en niet-vluchtige oliën zijn verschillend, maar beide leken veelbelovend als middel om keverschade te voorkomen.

Een aantal planten was dus effectief als poeder of als extract. Maar tot nu toe is er alleen gekeken naar de effecten van de botanische middelen op de plaaginsecten. Het indirecte effect van de middelen op de houdbaarheid van zaden door de effecten op de natuurlijke vijanden van de plaag werd ook onderzocht (hoofdstuk 5). Vier plantenpoeders en zes oliën met een bekend effect op de ogenbonenkever werden getoetst op hun mogelijk negatieve neveneffecten op de eiparasitoïde *Uscana lariophaga* en de larvale parasitoïde *Dinarmus basalis*. Alle behandelingen veroorzaakten een vermindering in parasitering door de eiparasitoïde en poeders van tabak en tephrosia beïnvloedden ook de ontwikkeling negatief. In een twee-keuze situatie in een lineaire olfactometer hadden de meeste oliën een afstotend effect op de eiparasitoïde. De larvale parasitoïde legde minder eieren als de bonen behandeld waren met plantenpoeders, maar de eieren die werden gelegd ontwikkelden normaal. In een Y-vormige olfactometer maakte deze larvale parasitoïde geen onderscheid tussen geuren van onbehandelde bonen en bonen behandeld met plantenpoeders. De poeders van tabak en tephrosia hadden sterkere negatieve effecten dan die van valse tabak en neem. In

onbehandelde bonenmonsters, verzameld in traditionele opslagfaciliteiten, kon geen van de behandelingen in het laboratorium de opbouw van een keverpopulatie verhinderen. Vierentwintig dagen na de behandeling waren de grootste aantallen kevers tevoorschijn gekomen uit bonen behandeld met poeders van tabak en tephrosia. Mogelijk werd dat veroorzaakt door het negatieve effect van de behandelingen op de parasitoïden. De botanische middelen die hier getest zijn, hadden negatieve effecten op de parasitoïden, maar poeders van neem en valse tabak zouden gecombineerd kunnen worden met biologische bestrijding door de larvale parasitoïde omdat ze effectief waren tegen de kever, maar geen ernstig negatief effect hadden op de parasitoïden.

De mogelijke neveneffecten op de menselijke consumenten van de bonen werden ook onderzocht. Uit het feit dat de producten al van oudsher gebruikt werden zou afgeleid kunnen worden dat ze redelijk veilig zijn, of dat behandeling van de bonen met deze middelen ten minste minder erg is dan het gebrek aan voedsel dat zou ontstaan als de bonen onbehandeld bleven. Echter, omdat plantenproducten complexe en variabele mengsels zijn met vele componenten, zou een gedetailleerde beschrijving van de giftigheid zeer uitgebreid onderzoek vergen. Om toch de mogelijke neveneffecten van botanische insecticiden te kunnen beoordelen, werd de beschikbare literatuur geraadpleegd over het bekendste voorbeeld van een bron van zulke middelen: de neemboom (hoofdstuk 6). Er werd een overzicht gemaakt van de toxicologische publicaties over experimenten met mensen en dieren waarin verschillende preparaten van neem oraal werden toegediend. Voordelige effecten, zoals bloedsuikerverlaging, anti-parasitaire, onstekingsremmende, anti-maagzweer en leverbeschermende effecten werden vaak beschreven, maar ook giftige effecten werden gevonden. Voor alle preparaten werden reversibele effecten op de voortplanting gemeld voor mannelijke en vrouwelijke zoogdieren. Uit de beschikbare gegevens werden veilige doses berekend en vergeleken met de inname van residuen van de neempreparaten op behandelde bonen. Mits verse delen van de neemboom gebruikt worden, zodat ze vrij zijn van aflatoxine producerende schimmels en de residuen van het insecticide goed van de bonen gewassen worden vóór de bereiding, is het gebruik van neem preparaten veilig te noemen.

Al het werk dat tot nu toe besproken is, werd uitgevoerd in het laboratorium, maar de effecten van de botanische insecticiden zouden anders kunnen zijn als ze gebruikt worden in de traditionele opslagstructuren. Daarom werd het effect van de plantenproducten op de ogenbonenkever bekeken in een veldproef in Benin (hoofdstuk 7). Zaden werden gemengd met fijngemalen droge klei en drie vluchtige oliën en vervolgens opgeslagen in luchtdichte jerrycans en blikken. Peulen werden behandeld met bladpoeder van twee planten en opgeslagen in traditionele opslaghutjes van palmbladeren. De schade door kevers werd voor en na opslag geanalyseerd. De toegepaste behandelingen konden de schade niet helemaal voorkomen, maar door de behandeling met oliën waren er minder bonen met kevergaten, en het percentage niet-aangetaste bonen en het gewicht van een liter bonen waren hoger. De

bonen kiemden het best als ze waren opgeslagen met basilicumolie. Bladpoeder van de bittere kalebas was effectief tegen gewichtsverlies van de opgeslagen peulen, terwijl *Ficus exasperata* (ficus) ook het percentage aangetaste bonen en het aantal uitgekomen kevers verlaagde en er na behandeling met deze plant meer parasitoïden waren. Laboratoriumonderzoek naar het effect van de oliën op de ontwikkeling van de kever en op de kieming van de bonen, liet geen effecten zien van de behandelingen. Met de niet-vluchtige oliën en de slurries zijn nog geen proeven gedaan in het veld.

Als de bonen beschermd worden tegen de kevers door middel van geïntegreerde gewasbescherming, moeten de nadelige effecten van de plantenproducten op de parasitoïden afgewogen worden tegen hun remmende werking op de kever. Het is al veelbelovend dat in alle monsters in de veldproeven tekenen van parasitering aangetroffen werden.

Met de hulp van boeren en gebaseerd op hun eeuwenoude gewoontes werden traditionele methoden voor bescherming van opgeslagen zaden getoetst. Gedurende de zoektocht naar botanische beschermingsmiddelen bleek dat er tussen de van oudsher gebruikte planten effectieve voorbeelden te vinden zijn (tabel 1). Het feit dat niet alle gebruikte planten in de proeven effectief waren, kan gelegen hebben aan de toegepaste dosis die dan te laag was, of aan het feit dat het plantenmateriaal tenminste enkele maanden oud was voor het werd getest in het laboratorium. Het is ook mogelijk dat een bepaalde plant, als deze onder andere condities gegroeid was, wel een effect gehad zou hebben. Bewijs hiervoor werd geleverd doordat voor sommige planten monsters uit verschillende regio's getest werden. De resultaten lieten altijd verschillen zien tussen deze monsters. Concluderend kan gezegd worden dat er belangrijke verschillen waren in beschermende effectiviteit tussen de van oudsher gebruikte plantenproducten.

Bovendien, zoals blijkt uit de veldproeven, kunnen de resultaten uit het laboratorium niet altijd een beeld geven van wat er in het veld zal gebeuren. Het monster van ficusbladeren dat in het laboratorium getest werd, had geen enkel effect op de kever terwijl deze plant in het veld effectiever was dan de bittere kalebas die in het laboratorium een afstotend effect had op de kever. Dit afstotende effect van bittere kalebas in een twee-keuze situatie was duidelijk, maar in het veld was het niet zo uitgesproken dat de kevers inderdaad de opslag verlieten, ook al hadden ze daar wel de mogelijkheid toe. Als er een onbehandelde bonenvoorraad nabij geweest was, was dit effect wellicht wel duidelijk.

De plantenproducten die in het laboratorium getest werden op onaangetaste bonen konden slechts in enkele gevallen aantasting voorkomen en als ze getest werden op reeds aangetaste bonen konden de producten de groei van de keverpopulatie niet helemaal remmen. In veel gevallen groeide de keverpopulatie wel langzamer op behandelde bonen, wat resulteerde in minder schade. Op de markt in Benin kochten klanten doelbewust bonen die een beetje aangetast waren en waarop een paar insecten te vinden waren om er zeker van te zijn dat de bonen niet behandeld waren met zeer giftige chemische middelen (pers. observatie). De

behandeling van bonen met plantenproducten zal waarschijnlijk zo'n beeld geven, met weinig kevergaten en enkele insecten aanwezig in de opslag.
Uit de resultaten gepresenteerd in dit proefschrift kan geconcludeerd worden dat het gebruik van bepaalde plantenmaterialen ter bescherming van opgeslagen ogenbonen in Benin haalbaar is. De giftigheid, resteffecten, houdbaarheid en de effecten in het veld behoeven nog nader onderzoek. De methodes zouden ook toegepast kunnen worden voor andere opgeslagen zaden.

Résumé et conclusions

Les plantes, en d'autres termes les productrices de métabolites secondaires, pourraient être utilisées pour la protection d'autres plantes et leurs produits contre les dégâts causés par les insectes. Spécialement lorsque d'autres moyens de protection ne sont pas disponibles, les plantes qui interviennent négativement sur le comportement et la reproduction des insectes peuvent être utiles pour la protection des végétaux. Cette thèse se concentre sur la protection du niébé stocké (*Vigna unguiculata*) contre le coléoptère du niébé *Callosobruchus maculatus* à l'aide de produits végétaux.

Le niébé est cultivé partout dans les zones tropicales et surtout en Afrique de l'Ouest où il est l'une des cultures les plus importantes pour les paysans à faibles moyens. Ce haricot est riche en protéine et c'est pourquoi il est connu comme 'la viande des pauvres'. Le coléoptère du niébé est présent partout où le niébé est cultivé et il n'a besoin que des semences mures pour sa reproduction. Ce coléoptère pond ces œufs sur la surface du haricot et le développement de la larve et de la pupe a lieu à l'intérieur de la graine d'où le coléoptère adulte émerge. Pour cette raison, ce coléoptère se plaît très bien dans des structures de stockage où les graines sont conservées jusqu'au moment où l'on en a besoin pour la consommation, la vente ou le semis. Les graines de niébés fortement attaquées ne sont pas aptes à la consommation humaine, ne valent rien sur le marché et ne germent plus. En Afrique de l'Ouest, les moyens pour la protection des végétaux contre les insectes nuisibles sont souvent limités et les cultivateurs du niébé n'ont que des moyens limités pour éviter les dommages causés par ces insectes. Cette thèse expose les travaux de recherche sur les moyens de lutte peu onéreux, à risques limités et disponibles aux paysans du Bénin en Afrique de l'Ouest pour empêcher ou réduire la reproduction du coléoptère du niébé au stockage. La démarche du travail de recherche est exposée dans la figure 1.

Dans une revue de la littérature spécialisée sur la protection des graines stockés à l'aide des plantes (partie I de cette thèse), les produits végétaux sont catalogués et discutés en fonction de leurs effets, de leurs avantages et désavantages spécifiques lorsqu'ils sont utilisés en poudres, cendres, huiles essentielles ou non-essentielles ou extraits. Il apparaît que jusqu'à lors il n'y avait pas de tests standardisés et une classification selon l'efficacité des espèces de plantes ne pouvait pas être donnée. Les plantes provenaient de diverses régions du globe et les essais qui ont été faits pour examiner leurs effets sur le coléoptère variaient pour tous les paramètres.

Le reste du travail s'est concentré sur la protection du niébé stocké contre son insecte spécialisé à l'aide des plantes qui sont utilisées traditionnellement à cette fin au Bénin. Le succès reproductif du coléoptère du niébé peut varier avec le lieu d'origine du coléoptère et avec les variétés de la plante hôte. Les expériences sur l'effet nocif pour les insectes sont préférablement réalisées avec les coléoptères les plus prolifiques sur la variété de niébé la plus susceptible. Afin de trouver les meilleurs coléoptères et la variété la plus convenable,

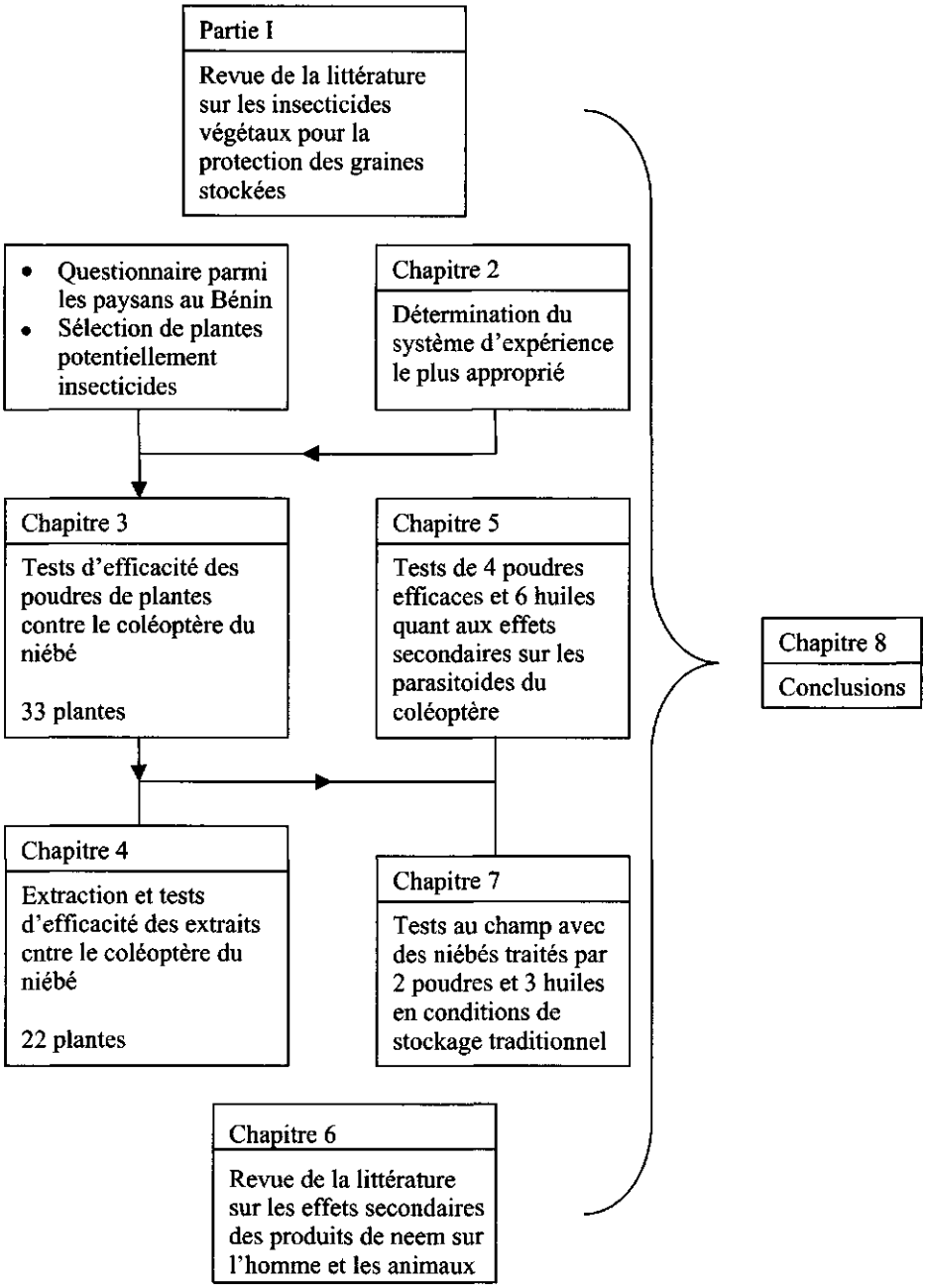


Figure 1: Schéma selon lequel les questions de recherche ont été résolues

dans chapitre 2 les paramètres reproductifs pour trois lignées de coléoptères originaires de différentes parties de l'Afrique de l'Ouest ont été comparées sur deux variétés de niébé susceptibles. Toutes les lignées de coléoptères ont été testées dans une situation sans choix et dans une situation à deux choix. Des différences importantes entre les lignées de coléoptères n'ont pas été observées. La période de développement de l'œuf jusqu'à l'adulte était plus longue sur la variété de niébé Kpodjiguegue. Dans un test à deux choix, les coléoptères ont montré une forte préférence pour la variété Californian blackeyed quant à la ponte de leurs œufs. Pour les expériences suivantes des coléoptères béninois ont été utilisés sur la variété de niébé préférée Californian blackeyed pour tous les tests faits au laboratoire.
Dans ce système expérimental les effets des plantes répulsives pour le coléoptère ou interférant avec sa reproduction ont été examinés. Dans un premier temps une enquête à été faite parmi les paysans au Bénin pour découvrir quelles espèces de plantes ils utilisaient traditionnellement pour la protection de leurs haricots stockés. Les plantes les plus souvent nommées ont été collectées et les poudres du matériel sec de 33 plantes ont été testées au laboratoire quant à leurs effets répulsifs et toxiques contre le coléoptère du niébé (chapitre 3, le Tableau 1 donne le résumé des résultats). Les effets sur le développement et la reproduction ont été mesurés dans une situation sans choix. La poudre des feuilles du tabac (*Nicotiana tabacum*), du téphrosia (*Tephrosia vogelii*) et de *Securidaca longepedunculata* ont réduit le nombre de coléoptères émergeant des graines traitées. L'effet répulsif a été testé en observant des femelles du coléoptère exposées aux graines traitées et non-traitées dans un olfactomètre linéaire. Les poudres des feuilles de clauséna (*Clausena anisata*), du dracaéna (*Dracaena arborea*), téphrosia, de la gourde amère (*Momordica charantia*) et du faux tabac (*Blumea aurita*) étaient les plus répulsives pour les coléoptères.
Pour analyser si les effets répulsifs et toxiques pouvaient être améliorés, les poudres qui se sont montrées efficaces ont été extraites avec de l'eau chaude (chapitre 4). L'appareillage pour cette extraction était simple et l'eau était choisie comme solvant pour que l'extraction puisse facilement être réalisée à grande échelle avec peu d'investissement en Afrique. Les extractions ont abouti à treize huiles essentielles, huit slurries et deux huiles non-essentielles. Ces extraits ont tous été testés quant à leurs effets répulsifs et toxiques. L'application des huiles essentielles réduisait souvent l'oviposition des coléoptères sur les graines traités. Les œufs sur des graines traitées avec les huiles essentielles de *Cymbopogon nardus* et de *C. schoenanthus* ne se sont pas développé jusqu'au stade adulte. Des effets répulsifs ont été observés pour les huiles essentielles de clauséna, herbe de citron (*C. citratus*), herbe citronnelle, un mélange de l'herbe de citron et l'herbe de citron de l'Inde de l'est (*C. flexuosus*), marubio (*Hyptis spicigera*), tagète (*Tagetes minuta*) et deux échantillons différents de l'huile du basilic (*Ocimum basilicum*). Les huiles non-essentielles n'étaient pas répulsives et n'avaient pas d'effet sur le nombre d'œufs pondus, mais le développement de ces œufs était inhibé en particulier avec l'huile de neem (*Azadirachta indica*). Aucune des slurries n'ont eu d'effet toxique sur les coléoptères. Seules les slurries extraites de la papaye

Tableau 1 : Résultats des tests au laboratoire et au champ avec des plantes utilisées traditionnellement pour la protection du niébé stocké contre le coléoptère du niébé.

Plante	Poudre	Extraction	Extrait	Parasitoides	Champ
Annona muricata	--	*	*	*	*
Annona senegalensis a[1]	TA	*	*	*	*
Annona senegalensis b[1]	R	*	*	*	*
Azadirachta indica	*	Huile	T	O	*
Azadirachta indica a[1]	A	*	*	*	*
Azadirachta indica b[1]	*	*	*	Ol	*
Azadirachta indica t[1]	A	*	*	*	*
Azadirachta indica z[1]	--	Slurrie	A	*	*
Blumea aurita	R	Huile	A	Ol	*
Capsicum frutescens	A	Slurrie	--	*	*
Carica papaya	R	Slurrie	R	*	*
Chamaecrista nigricans	A	*	*	*	*
Clausena anisata a[1]	TR	Huile	--	*	*
Clausena anisata b[1]	*	Huile	R	--	C
Combretum micranthum	R	Slurrie	--	*	*
Crateva religiosa	A	*	*	*	*
Cymbopogon citratus	--	Huile	TR	*	*
Cymbopogon MIX	*	Huile	R	O	C
Cymbopogon nardus	*	Huile	TR	O	*
Cymbopogon schoenanthus	*	Huile	T	--	*
Dracaena arborea a[1]	R	*	*	*	*
Dracaena arborea m[1]	R	Slurrie	R	*	*
Ficus exasperata	A	*	*	*	C
Helianthus annuus	*	Huile	T	*	*
Heliotropium indicum	R	*	*	*	*
Hyptis spicigera a[1]	A	Huile	R	*	*
Hyptis spicigera b[1]	--	*	*	*	*
Hyptis suaveolens	TA	*	*	*	*
Iboza multiflora	R	*	*	*	*
Khaya senegalensis	--	*	*	*	*
Momordica charantia	R	Slurrie	--	*	C
Moringa oleifera	T	*	*	*	*
Nicotiana tabacum	TR	Huile	--	OL	*
Ocimum basilicum b[1]	--	Huile	R	--	C
Ocimum basilicum t[1]	*	Huile	TR	*	*
Opilia celtidifolia	--	*	*	*	*

Plante	Poudre	Extraction	Extrait	Parasitoides	Champ
Pergularia daemia	--	*	*	*	*
Securidaca longepedunculata a[1]	A	*	*	*	*
Securidaca longepedunculata b[1]	TA	Slurrie	--	*	*
Tagetes minuta	--	Huile	R	*	*
Tephrosia vogelii	TR	Slurrie	R	OL	*

1: abréviation de la région d'origine des plantes, voire chapitres 3 & 4
-- = pas d'effet mesuré
* = pas examiné
A = attractif
C = testé et efficace au champ
L = nocif (ou l : non nocif) pour le parasitoide larvaire du coléoptère
O = nocif pour le parasitoide des œufs du coléoptère
R = répulsif
T = toxique : effet négatif sur la reproduction

(*Carica papaya*), du dracaéna et du téphrosia étaient répulsives. Les huiles essentielles et non-essentielles, ont été extraites aisément du matériel végétal et elles ont montré des effets prometteurs comme protecteur des niébés stockés. Cette extraction simple n'améliorait pas toujours améliorer l'efficacité des poudres de plantes, mais si de l'huile était obtenue grâce à l'extraction, l'effet contre les insectes était augmenté. Les mécanismes d'action des huiles essentielles et non-essentielles sont différents mais les deux paraissent prometteuses comme moyen pour prévenir les dommages des coléoptères.

Un nombre important de plantes était efficace en poudre ou en extrait. Jusqu'à maintenant, uniquement l'effet de ces produits végétaux sur l'insecte nuisible avait été examiné. L'effet indirect des poudres végétales sur la conservation des graines à cause de leur impact sur les ennemies naturels du coléoptère a été étudié au chapitre 5. Quatre poudres de plantes et six huiles ayant un effet connu sur le coléoptère de niébé ont été testées quant à leurs éventuels effets secondaires négatifs sur le parasitoide d'œufs *Uscana lariophaga* et le parasitoide des larves *Dinarmus basalis*. Tous les traitements ont causé une diminution du taux de parasitisme et les poudres de tabac et du téphrosia avaient un effet négatif sur le développement des parasitoides. Dans une situation à deux choix, dans un olfactomètre linéaire, les huiles avaient un effet répulsif sur le parasitoide des œufs. Le taux de parasitisme par le parasitoide larvaire était plus bas après traitement des graines avec les poudres des plantes, mais les œufs qui étaient pondus se développaient normalement. Dans un olfactomètre de forme de Y, ce parasitoide larvaire ne choisissait pas entre l'odeur des graines traités avec les poudres de plantes et celle des graines non-traités. Les poudres du tabac et du téphrosia avaient un effet négatif plus important que les poudres du faux tabac et

du neem. Au laboratoire des échantillons qui avaient été collectionnés dans des structures de stockage traditionnels, ayant été traité avec des poudres de plantes, aucun des traitements ne pouvait prévenir l'augmentation de la population du coléoptère. Vingt quatre jours après le traitement, le nombre le plus important de coléoptères avait émergé des graines traitées avec les poudres du tabac et du téphrosia, probablement à cause de l'effet secondaire sur les parasitoides. Les produits végétaux testés ici influençaient négativement les parasitoides, mais les poudres de neem et du faux tabac peuvent être compatibles avec la lutte biologique par le parasitoide larvaires.

D'autres effets secondaires des poudres insecticides sur les consommateurs humains du niébé ont été également étudiés. Le fait que les produits sont utilisés traditionnellement peut suggérer qu'ils sont relativement inoffensifs ou bien que le traitement des graines avec ces produits cause moins de problèmes que la famine quand les graines ne sont pas traitées. Toutefois, comme les produits végétaux sont tous des mélanges complexes et variable, comportant de nombreux éléments, une description détaillée de leur toxicité exige des études approfondies. Afin d'analyser les effets secondaires éventuels des insecticides végétaux sur l'homme, la littérature disponible pour l'exemple le mieux connu d'un tel insecticide, l'arbre de neem, a été consultée dans le chapitre 6. Une étude toxicologique a été faite des expériences sur l'homme et les animaux après administration orale de différentes préparations à base de neem. Des effets favorables comme la baisse de sucre dans le sang, les effets contre parasites, contre l'inflammation, contre l'ulcère et hepatoprotectifs ont été souvent rapportés, mais des effets toxiques ont été observés également. Pour toutes les préparations, des effets réversibles sur la reproduction des mammifères mâles et femelles ont été observés. A partir des données disponibles, des doses inoffensives ont été calculées et comparées avec l'ingestion des résidus des préparations de neem sur les haricots mangés. Il faut que les parties de l'arbre de neem utilisées pour la préparation soient fraîchement récoltées, pour réduire le risque d'infestation de moisissures produisant des aflatoxines. Si les graines sont bien lavées avant la cuisson pour éliminer les résidus de l'insecticide, l'usage des produits du neem comme insecticides sont inoffensif.

Le travail rapporté jusqu'ici a été réalisé en laboratoire, mais les effets des insecticides végétaux peuvent être différents quand ils sont appliqués dans des structures de stockage traditionnels. Pour cette raison, les effets des produits végétaux ont été analysés au champ également (chapitre 7). Des graines, mélangées avec de l'argile sèche moulue et trois huiles essentielles ont été stockées dans des bidons et des fûts hermétiques. Des gousses traitées avec la poudre de feuilles de deux espèces de plantes ont été stockées dans des cabanes traditionnelles faites de feuilles de palmiers. Les dommages des coléoptères ont été jugés avant et après stockage. Les traitements appliqués ne pouvaient pas prévenir les dommages, mais après traitement avec les huiles, moins de graines avaient de trous d'émergence de coléoptères et le pourcentage de graines non-infestées et le poids d'un litre de graines étaient plus grand. La germination des graines stockées était la meilleure après traitement avec

l'huile de basilique. La poudre des feuilles de la gourde amère était efficace contre la perte de poids des gousses stockées tandis que la poudre de ficus (*Ficus exasperata*) diminuait le pourcentage de graines infestées et le nombre de coléoptères émergeant et un nombre plus important de parasitoides ont émergé. Les expériences au laboratoire sur l'effet des huiles sur le développement du coléoptère et sur la germination des graines n'ont pas révélé ces effets. Pour les huiles non-essentielles et pour les slurries, les tests n'ont pas été effectués au champ.

Lorsqu'on veut protéger les graines contre le coléoptère du niébé dans une approche de lutte intégrée (IPM), les effets défavorables des produits végétaux sur les parasitoides doivent être comparés avec les effets négatifs sur le coléoptère. Il semble prometteur que dans l'expérience au champ il y avait des parasitoides dans tous les échantillons.

Avec l'aide des paysans et leurs pratiques séculaires, les méthodes traditionnelles ont pu être testées. La recherche sur les produits végétaux utilisés traditionnellement, a montré que certains étaient efficaces contre les coléoptères. Le fait qu'ils n'aient pas tous montrés des effets dans nos expériences, peut être due à la dose trop basse utilisée ou parce que les plantes avaient été collectées quelques mois avant usage au laboratoire. Il est possible également que les conditions dans lesquelles les plantes ont poussé puissent influencer leurs effets insecticides. Ceci a été démontré pour quelques plantes utilisées dans les tests dont il y avait plusieurs échantillons d'origines différentes. Les résultats de ces tests étaient toujours différents en fonction des échantillons. Des différences importantes d'efficacité protecteur ont été observées entre les produits végétaux utilisés traditionnellement.

De plus, comme cela a été démontré lors des tests au champ, les résultats obtenus au laboratoire ne peuvent pas toujours prédire ce qu'il se passera au champ. L'échantillon de ficus qui a été testé au laboratoire n'avait aucun effet sur le coléoptère, alors qu'au champ cette plante était plus efficace que la gourde amère qui, au laboratoire, avait montré un effet répulsif. L'effet de la gourde amère dans une situation a deux choix au laboratoire était évidant, mais au champ l'effet n'était pas si prononcé : le coléoptère ne sortait pas de la structure de stockage, même s'il en avait la possibilité. S'il y avait eu un stockage de niébé non-traité à proximité, l'effet aurait pu être plus évident.

Les produits végétaux testés au laboratoire avec des niébés non-infestés ne pouvaient que dans quelques cas prévenir l'infestation et quand ils étaient testés avec des niébés déjà infestés les produits n'arrêtaient pas complètement la croissance de la population du coléoptère. Dans la plupart des cas, la population du coléoptère croissait plus lentement sur les niébés traités, entraînant un nombre de graines infestés moins important. Sur le marché au Bénin, les gens achetaient délibérément des haricots peu infestés par les coléoptères mais avec quelques insectes présents entre les graines pour être sûr que les graines n'avaient pas été traités avec des produits chimiques nocifs (observation pers.). Le traitement avec les produits végétaux donnera probablement de tels résultats : des graines avec peu de trous et quelques insectes présents dans la structure de stockage.

Des résultats présentés dans cette thèse, il peut être conclu que l'usage de matériel des plantes insecticides sélectionnées pour la protection du niébé stocké au Bénin est applicable. La toxicité, les effets des résidus, la persistance et les effets au champ, spécialement pour les slurries doivent être examinés plus profondément. Les méthodes peuvent être applicables également pour des autres espèces de graines.

Nawoord

O ja, een nawoord om alle mensen die geholpen hebben bij het tot stand komen van dit boekje heel hartelijk te bedanken. Niet onbelangrijk, want dat wordt toch het meest gelezen en o wee als iemand er niet in staat.
Wel, het schrijven van een proefschrift over boontjes gaat niet over rozen, heeft heel wat voeten in de aarde en er zitten veel figuurlijke haken en nog veel meer letterlijke ogen aan. Vier jaar, 614 keer kevers kweken, 18 entomologen zien promoveren, 0 dagen ziekte, 437 9-cm petrischalen, 5 keer naar Benin, 561 5-cm petrischalen, 1 computer en ontiegelijk veel kevertjes waren nodig. Toch ligt het hier nu klaar te zijn en dat was niet gelukt zonder de bezielende begeleiding van Marcel Dicke die tijdens het project steeds meer professor werd. Hij las en bestreepte met zijn potlood al die hoofdstukken in al die boekjes. Steeds was het commentaar iets als "Sara, ik heb dit ms met plezier gelezen. Er zitten veel goede dingen in, maar ..." En meestal had hij dan gelijk.
Arnold van Huis deed het recht voor mijn raap met pen of fijnschrijver, en ook met de telefoon en de e-mail. Het heeft enorm geholpen dat er af en toe een doctorstitel gebruikt kon worden in de communicatie. Of ze nou low-resource zijn of resource-poor, de boeren in Afrika en ik hebben veel geleerd en veel voordeel genoten van zijn ervaring en werk.
Het meest, hoewel ik dat niet statistisch kan bewijzen, heeft Joop van Loon geleden onder alle eerste versies en de dagelijkse beslommeringen op het lab. Functioneringsgesprekken, Beninese geldproblemen, studentenverslagen, we sloegen ons er samen doorheen.
Bien sûr, ce projet ne serait rien sans les paysans et les partenaires africains du projet niébé. Sans eux je n'aurais pas eu mes bruches, ni mes plantes, ni la plupart des huiles essentielles, ni la plupart des données pour les chapitres 5 et 7, ni mes très bonnes expériences dans le joli pays qui s'appelle le Bénin. Le grand chef au Bénin, dr. Kossou, a toujours arrangé tout pendant mes séjours là bas et pendant mes séjours ici. La coopération était bonne. Pourtant, l'aide des autres était indispensable. Sabin was the dedicated secretary who wanted to, and will learn to speak fluent English. Darius m'a emmené partout dans son grand véhicule climatisé. J'admire sa patience. L'équipe du labo du FAST et tous les représentants dans les villages ont toujours fait leurs mieux pour m'aider et pour faire ce que nous aux Pays-Bas avions inventé. Merci à tous, surtout à Louba, Albert, Zacharie, Hervé, Désiré, Amélie, Mamatou, Euloge, Antonio, Bernardin, Eli et tous ceux dont je ne connais pas les noms. Puis j'aimerai remercier l'équipe du SPV à Porto-Novo, Bénin et les équipes au Togo, à Burkina Faso et au Niger pour leurs accueils chaleureux.
Beaucoup de travail a été fait par mes étudiants dévoués. Antonio et Cécile ont obtenu des bons résultats que j'ai pu utiliser dans les chapitres 5 et 4 de cette thèse. Merci mille fois, les francophones. Misschien wel de meest toegewijde student was Iertje Baumgart die zich maandenlang semi-vrijwillig opsloot in de klimaatcel om te tellen. Kevers, eieren, dagen, bonen, alles heeft ze geteld en zo kregen we een beeld van welke planten als poeder effectief

waren tegen de kevers. Zonder haar was hoofdstuk 3 een veel zwaardere bevalling geworden voor mij.
Deel 1 van dit proefschrift was nooit in deze vorm gepubliceerd als er niet geknokt was voor het voortbestaan van de serie. Hiervoor heeft professor Van der Maesen zich met uiteindelijk succes ingezet en daarvoor zou ik hem graag bij deze nogmaals danken.
Als je dan resultaten hebt en weet welke planten de kevers binnen de perken kunnen houden, dan is het tijd om te kijken of de mensen die dan in ieder geval bonen hebben om te eten, niet ook doodgaan aan de behandeling. Daartoe was ik aangewezen op buitenlaboratoriale hulp die ik vond bij de divisie toxicologie. Uiterst prettig was de samenwerking met professor Ivonne Rietjens, Marelle Boersma en Gerrit Alink die ervoor zorgden dat ik als leek toch een toxicologisch verantwoord overzicht heb kunnen geven van de neveneffecten van botanische insecticiden. Het schrijven van dit hoofdstuk was ook een eind moeilijker geweest zonder de artikelen die ik via mijn privé copieerservices kon verkrijgen. Kobus en Harma, mijn dank is eindeloos.
Maar werken is niet half zo leuk zonder collega's om je heen. Clemens, die samen met mij in de bonen was, dank ik voor de nuttige tips, foto's, adressen, overleg, bestelling van de bonen enz. Mijn kamergenoten waren goud waard bij het opnemen van de telefoon, bij het leren van Chinees en bij het delen van frustraties over proefschriften en wat dies meer zij. Met de buren deelde ik lief en leed, maar vooral koffie. Zonder Olivier waren het résumé en de conclusions veel minder Frans geworden. Feestciegenoten en voorzitter Yde dank ik voor Sint en andere feesten die bij de voorpret minstens zo leuk waren als op de dag zelf. Gelukkig heb ik al die jaren aan het voordragen van Sintgedichten weten te ontkomen. Peter dank ik voor de koekjes en Wouter voor de narcissen en de goede gaven uit eigen tuin. Ook degenen die hun naam hier nu niet zien staan, zal ik missen om de koffie-, en lunchpraat en om andere socialere aspecten van de wetenschap.
Ontspanning of ontsnapping, het is soms niet duidelijk, maar zonder Poco meno mosso, BKK, Vivavoce, Onvacie, WKK, NJN, WSKOV, Yvonne en Sjoukje was er weinig te hobbyen, zonder familie weinig kerst te dineren of paas te ontbijten, zonder ouders, broer, zus en aanhang weinig stof tot heftige discussies of gemene gedichten en zonder Vrlendje, die niet met zijn naam in dit hoofdstuk wil, maar die dat misschien wel het meest van allen verdient, was het leven minder aangenaam. Allemaal enorm bedankt.
Zo, en dan ga ik nu maar eens trombone spelen, of nee cello, of zal ik die stapel kranten maar eens te lijf gaan, of mijn fiets uit de schuur halen, of een boek lezen ... Hopelijk is er leven na een promotie.

Gr
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Curriculum vitae

Sara Boeke werd geboren op de laatste zondagmiddag van april in 1974 te Groningen. Ze doorliep met succes alle klassen van het Kwetternest en de Roelof van Wienesse school in Wijdenes en ze behaalde haar VWO diploma aan de Openbare Scholengemeenschap in Hoorn in 1992. In datzelfde jaar begon ze met haar studie Moleculaire Wetenschappen aan de Landbouwuniversiteit in Wageningen. In de doctoraalfase van die opleiding deed ze bij de vakgroep plantenfysiologie vijf maanden onderzoek aan de tolerantie van het fotosynthetisch systeem van planten voor UV-B straling. Vervolgens ging ze naar de Université de Rouen in Frankrijk om gedurende vijf maanden onderzoek te doen aan het gedrag van polymeermoleculen na substitutiereacties. Als laatste afstudeervak onderzocht ze gedurende vier maanden bij de vakgroep Microbiologie in Wageningen welke bacteriën er aanwezig zijn in de bodem van 'rice paddies'. In 1997 behaalde ze haar ingenieursdiploma. Vervolgens werkte ze drie maanden als laboratoriumassistent bij de Plantenziektekundige Dienst en begon ze in mei 1998 aan haar werk als AIO bij het Laboratorium voor Entomologie van de Landbouwuniversiteit in Wageningen. Het project, in samenwerking met diverse organisaties in Nederland en in Benin waaronder de Université Abomey Calavi in Cotonou heeft geleid tot het proefschrift dat u nu in handen heeft.