An analysis of potential resistance of the phytophagous mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) to four botanical pesticides

Sabrine Attia (1,2,3,4), Kaouthar Grissa Lebdi (2), Stéphanie Heuskin (3), Georges Lognay (3), Thierry Hance (1)

(1) Université Catholique de Louvain. Biodiversity Research Centre. Earth and Life Institute. Place Croix du Sud, 4-5. BE-1348 Louvain-la-Neuve (Belgium). E-mail: sabine_bio5@yahoo.fr
(2) Institut National Agronomique de Tunis. Laboratoire d’Entomologie-Acarologie. 43, Avenue Charles Nicolle. TN-1082 Tunis Mahrajene (Tunisia).

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**Description of the subject.** Synthetic acaricides have been widely used to manage *Tetranychus urticae*. Due to the excessive use of biocide and the associated problems of pesticide resistance and environmental pollution, there is an increasing demand for sustainable, environmentally-friendly control methods. Among the current alternative strategies aimed at decreasing the pest populations, the pesticides based on plant extracts are currently one of the most promising methods. Essential oils with acaricidal properties have been categorized as green pesticides because they are biodegradable and predominantly non-toxic to vertebrates.

**Objectives.** With an aim to reduce the use of synthetic pesticides, they represent a promising approach for eco-chemical control of mites.

**Method.** The aim of the present work was to analyze the risk of resistance emergence of *T. urticae* to repeated treatments with four plant extracts: *Deverra scoparia* Coss. & Durieu (Araliales: Apiaceae), *Hertia cheirifolia* (L.) Kuntze (Asterales: Asteraceae), *Santolina africana* Jord. & Fourn. (Asterales: Asteraceae) essential oils and garlic distillate *Allium sativum* L. (Asparagales: Alliaceae) after 20 generations.

**Results.** Repeated treatments with *S. africana* essential oil during 20 generations did not provoke an emergence of resistance while a low development of resistance was observed with *H. cheirifolia, A. sativum* and *D. scoparia* extracts.

**Conclusions.** The efficacy of these extracts against the two spotted spider mite and their low development of resistance make them a promising use for pest management.

**Keywords.** *Tetranychus urticae*, essential oils, pesticide resistance, pest management.

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Analyse de la résistance à quatre extraits de plantes chez l’acarien phytophage *Tetranychus urticae* Koch (Acari : Tetranychidae)

**Description du sujet.** Le développement des stratégies de lutte intégrée est en croissance depuis que de nombreux problèmes sont apparus suite à l’utilisation abusive et non raisonnée des pesticides de synthèse. Les extraits de plantes sont de plus en plus considérés, au sein des stratégies de lutte intégrée, comme des approches alternatives ou complémentaires aux traitements insecticides. Beaucoup d’huiles végétales essentielles montrent un large spectre d’activité contre les ravageurs.

**Objectifs.** Notre objectif est de tester si l’acarien *Tetranychus urticae* arrive à développer une résistance vis-à-vis des extraits naturels de plantes.


**Résultats.** Le traitement par *S. africana* ne montre aucune résistance, tandis qu’un faible développement de la résistance a été observé avec *H. cheirifolia, A. sativum* et *D. scoparia*.
1. INTRODUCTION

*Tetranychus urticae* Koch (Acari: Tetranychidae), the two spotted spider mite is a highly polyphagous pest that can devastate many crops (Migeon et al., 2007) of over 900 species of plant-hosts such as citrus, avocado, beans, cotton, apples, pears, plums and many other horticultural and ornamentals crops (Helle et al., 1985). It is especially dominant in intensive, high-yield cropping systems. It affects yields by feeding on leaves thereby reducing the area of photosynthetic activity (Helle et al., 1985; Rabbinge, 1985). When severe infestations, it causes leaf abscission. Spider mites are thus a major concern for growers worldwide in terms of damage and control cost and are therefore considered as one of the most important pests (Helle et al., 1985).

Synthetic acaricides are usually applied by farmers to reduce losses. However, the extensive use of these substances had led to the development of resistance. *Tetranychus urticae* pesticide-resistant populations have been reported in more than 40 countries in both greenhouses and open field crops (Georghiou et al., 1991; Stumpf et al., 2001; Van Leeuwen et al., 2007; Van Leeuwen et al., 2010). The rapid development of resistance in *T. urticae* is favored by its high reproductive potential, extremely short life cycle and arrhenotokous mating system (Helle et al., 1985; Van Leeuwen et al., 2005): for commercial acaricides, such as organophosphates, dicrof, organotins, failures in the chemical control of spider mites caused by resistance have been reported only a few years after their introduction (Jeppson et al., 1975; Cranham et al., 1985; Van Leeuwen et al., 2006). Another problem is the possible cross-resistance between active matters that put the availability of pesticides under further pressure (Van Pottelberge et al., 2009). Among current alternative strategies aiming at decreasing the use of chemical pesticides, ecochemical control based on plant-insect relationships is one of the most promising methods. Indeed, higher plants produce different bioactive secondary metabolites involved in their natural resistance to pathogens, insects and mites. Essential oil extract contains many of these compounds particularly secondary metabolites that are frequently concentrated in the leaves, bark, or fruit of aromatic plants (Isman et al., 2001; Kumar et al., 2011). Plant resistance to insect pests can result from the secretion of these essential oils (Regnault-Roger, 1997). For example, the resistance of Scotch pine *Pinus sylvestris* (conifers) to flat bugs *Aradus betulae* (Aradidae) was related to a high content of essential oil in the primary bark (Smelyanets et al., 1973). Another example is *Chrysanthemum cinerariaefolium* (Compositae) and *Chrysanthemum coccineum* that contain pyrethrin, a potent insecticide with a rapid knock down action (Ryan et al., 1988). The pulegone found in mint oils repels the German cockroach *Blatella germanica* (L.) (Praveena et al., 2011). These examples show that plant products may provide eco-chemical and biorational strategy to protect cereals, pulses and other agricultural commodities from losses by pests (Dubey, 2010; Attia et al., 2013).

However, the repeated application of essential oils for pest control could also potentially induce the acquisition of resistance. It is therefore necessary to test that possibility even if it is less likely that mites will evolve resistance to essential oils as they are a mixture of different active compounds. This low acquisition of resistance linked to the complexity of essential oil composition was already shown in insects (Liu et al., 2004) but has never been explored in mites. The purpose of this study is to analyze the emergence of the resistance of a *Tetranychus urticae* population to repeated applications of *Deverra scoparia* Coss. & Durieu, *Santolina africana* Jord. & Fourn., *Hertia cheirifolia* (L.) Kuntze essential oils and of a garlic extract, *Allium sativum* L. This selection of plants is based on previous works (Attia et al., 2011a; Attia et al., 2011b; Attia et al., 2011c; Attia et al., 2012) where we examined the effect of different concentrations of *S. africana*, *H. cheirifolia*, *D. scoparia* essential oils and garlic extract *A. sativum* against *T. urticae*. We showed that they caused significant *T. urticae* mortality after 24 h at low concentrations, with *LC*$_{50}$ values of 2.35 mg·l$^{-1}$ for *S. africana*, 3.43 mg·l$^{-1}$ for *H. cheirifolia*, 1.79 mg·l$^{-1}$ for *D. scoparia* and 7.49 mg·l$^{-1}$ for *A. sativum* (Attia et al., 2011a; Attia et al., 2011b; Attia et al., 2011c; Attia et al., 2012). In addition, fecundity decreased after treatments with sublethal concentrations of extracts corresponding for *D. scoparia* to 0.07, 0.09, 0.29 mg·l$^{-1}$, *H. cheirifolia* and *S. africana* oils to 0.064, 0.08, 0.26 mg·l$^{-1}$ respectively and for the garlic distillate, *A. sativum*, to 0.36 and 0.74 mg·l$^{-1}$ (Attia et al., 2011b; Attia et al., 2011c; Attia et al., 2012). In the present study, emergence of resistance experiments was performed on 20 generations. Our study is the first to evaluate the emergence of resistance to some natural biopesticides of one of the most important pest in the world, *T. urticae*. 

**Conclusions.** Ce travail a démontré l’efficacité de ces extraits contre *T. urticae*. De plus, le fait qu’ils induisent peu de résistance confirme leur valeur en tant qu’outil efficace pouvant être utilisé dans une lutte intégrée contre les ravageurs.

**Mots-clés.** *Tetranychus urticae*, huile essentielle, resistance aux pesticides, gestion des organismes nuisibles.
2. MATERIALS AND METHODS

2.1. Collection and maintenance of mites

_Tetranychus urticae_ were collected from infested plants in citrus orchards in Tunisia and transferred to a climate controlled room (26 °C, 50–60% RH, 16:8 [L:D]) in a laboratory at the Biodiversity Research Centre, UCL, Louvain-la-Neuve (Belgium) for more than 5 years without any contact with pesticides before the experiments. The population was reared on bean leaves (_Phaseolus vulgaris_) placed on moistened cotton in Petri dishes (Overmeer, 1985). Only young adult (24 h old) females were chosen for this bioassay.

2.2. Plant and essential oil extraction

Plants used for this study were collected locally in Tunisia, and were free of any pre-harvest chemical treatments (organic products such as Spirodiclofen and Fenbutatin oxide). The plants were freshly harvested and sorted for uniformity and absence of defects before being stored at -2 °C until the analysis and the tests.

Extracts of _A. sativum_ and _D. scoparia_, _S. africana_ and _H. cheirifolia_ oils were obtained from 3 kg of the aerial parts of the plants by hydrodistillation for 3 h using a Clevenger-type apparatus. Resulting oils were diluted 1:100 in absolute ethanol.

The essential oil yield of the plants used in the current study was 0.5% of the dry weight of _S. africana_, 0.7% of _H. cheirifolia_ and 0.5% of _D. scoparia_.

2.3. Toxicity bioassay

A group of 2,000 virgin young adult females _T. urticae_ (24 h) were randomly selected and then transferred to 40 fresh bean leaf discs (diameter = 35 mm) mounted with the adaxial side uppermost on moistened cotton in Petri dishes (90 x 15 mm), meaning 50 _T. urticae_ by leaf disc. A Potter spray tower producing a uniform deposit was used to spray individuals of _T. urticae_ after settling time on the leaf discs at a 1.4 bar pressure. Plants extracts were diluted in ethanol to obtain a homogenous spray film at 20 ± 2 °C. After this first selection (or treatments with extracts), the surviving _T. urticae_ were transferred back to untreated bean plants and the population was allowed to increase during one generation. The second selection (or treatment) was conducted after two generations: once again, a group of 2,000 young adult (24 h) females _T. urticae_ were randomly selected in the population coming from the surviving individuals of the last treatment. The tested _T. urticae_ were sprayed in the similar conditions as described before. In whole, 20 successive selections (10 months) were done each separated by two generations of _T. urticae_.

_Tetranychus urticae_ were treated with extracts of _A. sativum, D. scoparia, S. africana_ and _H. cheirifolia_ at the LC90 of 13.5, 5.07, 3.79 and 3.2 mg l⁻¹ of ethanol respectively to test the induction of resistance. These values were calculated from previous works (Attia et al., 2011b; Attia et al., 2011c; Attia et al., 2012) using concentrations that killed 90% of _T. urticae_, for each kind of extract respectively.

One control treatment was done and without any plant extracts but ethanol at the same concentration.

2.4. Statistical analyses

For each treatment, we calculated the number of surviving individuals on the 40 × 50 sampled every two generations. Our hypothesis was that, if there was no resistance, the number of surviving _T. urticae_ should not differ after each selection, meaning that the number of surviving _T. urticae_ induced by the first selection (that we called Nₙ) should be similar to the number of surviving _T. urticae_ induced by the second selection (N₁ₙ) and so on.

We calculated all the differences between all the N: N₁₋ₙ₁ (called Nₙ₋₁ₙ), N₁₋ₙ₂,… N₁₋ₙₙ₋₁, N₂₋ₙ₁, N₂₋ₙ₂,… N₂₋ₙₙ₋₁, Nₙ₋₁₋ₙ₂,… Nₙ₋₁₋ₙₙ₋₁. If there was no resistance, the median of all these differences should not statistically differ from 0. If there is resistance, the Nₙ should increase after selection and the median of the differences should be lower than 0. Our hypothesis was that: Ho: mediate of Nᵥᵥ₋ₙᵥ = 0 vs HA: mediate of Nᵥᵥ₋ₙᵥ ≠ 0.

As the calculated differences (called Nᵥᵥ₋ₙᵥ) were not normally distributed, we used a Wilkson Singed Rank test to compare the median of each treatment with the value “0”. To compare all the treatments between them, we used a Kruskall-Wallis test. Tests were performed using Graph Pad version 5.01 for Windows® (Graph Pad Software, San Diego, USA). All tests were applied under two-tailed hypotheses and the significance level p was set at 0.05.

3. RESULTS

3.1. Effects of the treatments on the resistance

The median of the calculated differences (Nᵥᵥ₋ₙᵥ) for each treatment was compared to the value 0 (Table 1, Figure 1): two treatments, the control and _S. africana_, were not statistically different from “0”. Thus, these two treatments did not induce any resistance in _T. urticae_ for this lap of time. The medians of Nᵥᵥ₋ₙᵥ of the three other treatments, _H. cheirifolia, D. scoparia_ and _A. sativum_ were statistically different from “0”, thus indicating the induction of resistance (Table 1, Figure 1).
Plant extracts resistance in *Tetranychus urticae*

**Table 1.** The calculated differences ($N_{s-all}$) for all the treatments. We used Wilkson Signed Rank Test to compare the medians of $N_{s-all}$ to the value “0” — *Différence entre les différents traitements calculée selon le test de Wilkson Rank.*

<table>
<thead>
<tr>
<th></th>
<th>D. scoparia</th>
<th>H. cheirifolia</th>
<th>A. sativum</th>
<th>S. africana</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>190</td>
<td>190</td>
<td>190</td>
<td>190</td>
<td>190</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>-79</td>
<td>-187</td>
<td>-255</td>
<td>-56</td>
<td>-3</td>
</tr>
<tr>
<td>Median</td>
<td>-32</td>
<td>-111</td>
<td>-142</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>15</td>
<td>-32</td>
<td>-25</td>
<td>82</td>
<td>2</td>
</tr>
</tbody>
</table>

Wilkson Signed Rank Test
- Sum of signed ranks (W) -8,209, -14,846, -15,553, 2,367, -868
- Sum of positive ranks 4,685, 1,650, 1,201, 10,067, 3,441
- Sum of negative ranks -12,894, -16,496, -16,754, -7,700, -4,309
- \( P \) value (two tailed) < 0.0001, < 0.0001, < 0.0001, 0.1133, 0.2791
- Significant (\( \alpha = 0.05 \))? Yes, Yes, Yes, No, No

### 3.2. Differences between the five treatments

Concerning the four plant extracts, there was a significant difference between all the treatments. The post-hoc tests showed no statistical differences between the treatment with *S. africana* oil and the control treatment (*Table 2*) nor between *H. cheirifolia* vs *A. sativum* extracts. The other post-hoc tests showed statistical differences between all other pairs of treatments. In comparison of the cumulative of the differences between all the \( N_s \) in function of the number of selection, no resistance was observed towards *S. africana* (0%); however a little resistance developed with *D. scoparia* oil (about 10%), *H. cheirifolia* oil (30%) and *A. sativum* extract (50%) (*Figures 1* and 2).

### 4. DISCUSSION

Our results showed that *T. urticae* can develop a resistance towards *D. scoparia*, *H. cheirifolia* and *A. sativum* extracts within 10 months and 20 selections, but quite limited. However no resistance was observed in the case of *S. africana* essential oil. The resistance depends on the mode of application and may suggest that the essential oil has to be first in contact with the cells (Rafii et al., 2007). In the case of *S. africana*, it is possible that *T. urticae* does not have the ability to detoxify some of the chemicals contained in the oil that have perhaps different mechanisms of action.

The mode of action of the essential oils is not yet well known but some authors suggest it may also be due to suffocation, by blocking the spiracles of pests (Chaubey, 2008). Indeed, it has been shown with petroleum-derived oils (Beattie et al., 1995) that heavy saturated oil fractions can penetrate a long distance into the tracheae. This fraction forms physical barriers to gaseous exchanges and provokes suffocation. With this hypothesis, the efficiency of essential oils should depend on their physical more than on their chemical characteristics.

However, we think that suffocation is not the mode of action of essential oils as most of their compounds are light, unsaturated and volatile ones. Moreover, it is known that suffocation is an extremely slow process. During our observations, *T. urticae* were killed 24 h after treatments with essential oils. Therefore, it is likely that other factors than suffocation are the prime cause of mortality.

Differences in chemical composition of oils may probably explain why plant extract differ in resistance induction (Attia et al., 2011b; Attia et al., 2011c; Attia et al., 2012).

In previous works, we observed that *S. africana*, *H. cheirifolia* and *A. sativum* are especially rich in...
oxygenated compounds with 94.83%, 78.99%, and 36.8% respectively while _D. scoparia_ extract is rich in hydrogenated compounds with 92.96%. The most abundant components in the _Santolina_ oil were terpinen-4-ol (54.96%), α-terpineol (14%) and borneol (8.37%). In fact, compounds as limonene, trans-chrysanthenol, borneol, carvenone, cis-jasmone and guaiol are specific to _S. africana_ oil (Attia et al., 2013). The results of Attia et al. (2012) showed that the presence of all these constituents was necessary to equal the toxicity of the natural oil of _S. africana_ against _T. urticae_ (Attia et al., 2012). Moreover, complexity of the mixture should increase the time needed for resistance development compared to a pesticide based on a single active ingredient. This phenomenon was observed in the case of the green peach aphids, _Myzus persicae_, that can develop resistance to azadirachtin, the major ingredient of neem, _Azadirachta indica_ A.Juss (insecticide) but can not develop resistance to a refined neem seed extract containing the same absolute amount of azadirachtin but with many other constituents present (Feng et al., 1995). Ryan et al. (1988) showed that six terpinoids corresponding to pulegone, gossypol, citral, linalool, bornyl acetate and cineole may act as plant defense compounds and may inhibit the enzyme acetylcholinesterase. The insecticide effect of

### Table 2

The calculated differences ($N_{s-all}$) for all the treatments. We used one Kruskal-Wallis test to compare all the treatments. Post-hoc tests Dunns compared all pairs of treatments — Différence entre les différents traitements calculée selon le test de Kruskal-Wallis et le test de Dunn.

<table>
<thead>
<tr>
<th>Dunn’s Multiple Comparison Test</th>
<th>Difference in rank sum</th>
<th>$P &lt; 0.05$</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deverra scoparia vs Hertia cheirifolia</td>
<td>170</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Deverra scoparia vs Allium sativum</td>
<td>210</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Deverra scoparia vs Santolina africana</td>
<td>-120</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Deverra scoparia vs Control</td>
<td>-110</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Hertia cheirifolia vs Allium sativum</td>
<td>34</td>
<td>No</td>
<td>ns</td>
</tr>
<tr>
<td>Hertia cheirifolia vs Santolina africana</td>
<td>-290</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Hertia cheirifolia vs Control</td>
<td>-280</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Allium sativum vs Santolina africana</td>
<td>-330</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Allium sativum vs Control</td>
<td>-320</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Santolina africana vs Control</td>
<td>8.5</td>
<td>No</td>
<td>ns</td>
</tr>
</tbody>
</table>

### Kruskal-Wallis test

- $P$ value: 0.0001
- Exact or approximate P value: Gaussian Approximation
- $P$ value summary: ***
- Do the medians vary significantly?: Yes
- Number of groups: 5
- Kruskal-Wallis statistic: 240

### Figure 2

The cumulative of the differences between all the $N_s$ in function of the number of selection — L’effet des différents extraits des plantes durant les 20 générations étudiées.
Plant extracts resistance in *Tetranychus urticae*

*Cedrus deodora* (Pinaceae) extracts to the pulse beetle *Callosobruchus analis* (Coleoptera: Bruchidae) and to the house fly *Musca domestica* (Diptera: Muscidae) was related to a high content of terpenes compounds diversified (Agarwal et al., 1988).

We still are, however, lacking information on the exact way of action of essential oil compounds, whereas it is well-known for synthetic pesticides (Dekeyser, 2005; Isman et al., 2006; Van Leeuwen et al., 2007; Van Nieuwenhuyse et al., 2009). The rapid toxicity from essential oils or their monoterpenes in insects and other arthropods points to a neurotoxic mode of action (Isman et al., 2006). In fact, the octopaminergic nervous system is considered to be the site of action of some compounds of essential oils in the control of the American cockroach, *Periplaneta Americana* (Enan, 2001), and fruit fly (Enan, 2005). Symptoms of hyperactivity followed by hypertension of the legs and abdomen culminating and immobilization (Enan, 2001). The same author pointed that many essential oil constituents such as monoterpenoids poison insects by blocking octopamine receptors (Enan, 2001; Enan, 2005). In fact, octopamine, synthetized from tyramine, is a biogenic monoamine structurally related to noradrenaline that acts as a neurohormone, a neuromodulator and may be a neurotransmitter in Arthropods. It is present in relatively high concentrations in neuronal systems as well as in non-neuronal tissues of most invertebrate species including Arthropods (Roeder, 1999).

The toxic effects of essential oils might be attributed to their major components, as well as other minor and/or trace compounds. The toxicity could vary with both the compounds of the plant extract and the sensitivity of the mite. It has been suggested that volatile oils, either inhaled or applied to the cuticle, act by means of their lipophilic fraction reacting with the lipid parts of the cell membranes, and as a result, modify the activity of the calcium ion channels (Buchbauer et al., 1994). At certain levels of dosage, the volatile oils saturate the membranes and show effects similar to those of local anaesthetics. They can interact with the cell membranes by means of their physio-chemical properties and molecular shapes, and can influence their enzymes, carriers, ion channels and receptors. The interaction of various components in the essential oil might be another important factor for the difficulty of resistance-induction. Future experiments are necessary to establish if similar process are involved in *T. urticae*.

Regarding the resistance against synthetic insecticides, three main metabolic enzyme groups are known to be involved: esterases, glutathione S-transferases (GST) and mono-oxygenases (Van Leeuwen et al., 2010). Dekeyser (2005) pointed out that several acaricides have been identified as mitochondrial respiration inhibitors; these include active ingredients as pyridazine, pyrazole, quinazoline, naphthoquinone, pyrrole, thiourea and pyrimidine chemistries. Others are growth inhibitors, such as benzyolphenyureas, tetrazines, tetronic acids and oxazolines (Dekeyser, 2005). Only tetronic acids like spiридicolofen, spiromesifen work via inhibition of lipid synthesis. Benzoylphenyurea’s and etoxazole work via inhibition of chitin synthase (Van Leeuwen et al., 2010). Others have unknown mode of action. Among growth inhibitors insecticides, spiридicolofen differs from others in its biochemical mechanism as it interferes with lipid biosynthesis, blocking the enzyme acetyl-CoA carboxylase that allows *T. urticae* to form important fatty acids (Dekeyser, 2005; Bretschneider et al., 2007). Finally, insecticides have been identified as complex III inhibitor such as Bifenazate, Halfenprox, Lubrocythrine, and Milbemecint. Their mode of action is reported by Dekeyser (2005): in fact they could block the nervous signal or induce repetitive discharge and prolonge the opening of sodium ion channels, or, finally, act as a GABA agonist (Dekeyser, 2005) By analogy, future experiments should be focused on the identification of the mode and site of action of the plants extracts tested here and the discovery of compounds affecting developmental processes in *T. urticae*.

For frequent applications, *S. africana* essential oil seems to be the best candidate in our study. *Santolina africana* oil may be used as alternative strategy against *T. urticae* to the heavy use of classical insecticides.

**Bibliography**


(39 ref.)