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EFFECT OF *POLYSCIAS FILICIFOLIA* BAILEY EXTRACTS ON THE BEHAVIOR OF THE LEAF MINER *CAMERARIA OHRIDELLA* DESCHKA AND DIMIC ON HORSE CHESTNUT TREES

ANNA TOMCZYK[1], DANUTA KROPCZYŃSKA[1*], ALEKSANDRA PTAK[1], MARIJA TITOVA[2], NIKOLAJ SHUMIŁO[3], ALEKSANDER ORESHNIKOV[3], ALEKSANDER NOSOV[2], AND MIROSŁAWA FURMANOWA[4**]

[1]*Department of Applied Entomology, Warsaw Agricultural University, ul. Nowoursynowska 159, 02–787 Warsaw, Poland,*
[2]*Plant Physiology Department, Moscow State University, 119992 Moscow, Russia*
[3]*Timiryazev Institute of Plant Physiology, Russian Academy of Science, Botanicheskaya St. 35, 127276 Moscow, Russia*
[4]*Department of Biology and Pharmaceutical Botany, Medical University of Warsaw, ul. Banacha 1, 02–097 Warsaw, Poland*

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Extract of *Polyscias filicifolia* suspension culture was used to reduce damage to horse chestnut (*Aesculus hippocastanum*) leaves by larvae of the horse chestnut leaf miner (*Cameraria ohridella*). The results showed the repellent effect of biomass extracts on *Cameraria ohridella* moths. The number of moths caught per day on sticky traps treated with extract was more than 50% lower than the control. The same extract on leaflets caused *C. ohridella* females to lay ~20% fewer eggs than on leaflets without extract. *P. filicifolia* extract had a repellent effect on female moths in laboratory conditions as well. Only single mines were observed on leaves treated with *P. filicifolia* extract, five times less than on control leaves. The data indicate that *P. filicifolia* extract can be used as a repellent for *C. ohridella* in springtime when the overwintering generation emerges from pupae.

Key words: *Polyscias filicifolia*, *Aesculus hippocastanum*, *Cameraria ohridella,* plant-derived in Secticides, suspension culture.

INTRODUCTION

The horse chestnut leaf miner *Cameraria ohridella* (Lep., Gracillariidae) is an invasive species of horse chestnut *Aesculus hippocastanum* L. which has spread very fast across Europe. The first report on the occurrence of this species came from Macedonia, near Ohrid lake (Simova-Timosic and Filev, 1985). The moth was described by Deschka and Dimic (1986) as a new species named *Cameraria ohridella*. Within a few years it spread across most Central and Western European countries, including Poland where it was recorded over its whole territory in 2002 (Kukuła-Młynarczyk and Hurej, 2004). Recent reports show its presence in the United Kingdom (Tilbury et al., 2004), Denmark (Karshold and Kristensen, 2003) and Ukraine (Akimov et al., 2003).

The horse chestnut leaf miner is the only representative of the genus *Cameraria* in Europe. Although it is now twenty years since the discovery of the pest in Macedonia, its origin is still unknown. Possible areas of its origin include Japan, northern China, eastern India, the Balkans and North America. Several species of the genus *Cameraria* are known to infest *Acer*, but only one has been found on different chestnut species in North America. Asiatic species live mainly on plants of the Fabaceae. Two species (*C. niphonica, C. acericola*) attack *Acer* in Japan (Kenis et al., 2004).

Cameraria ohridella attacks the white flowering chestnut tree *Aesculus hippocastanum*, which is commonly planted in town parks, along streets and in gardens in Europe. Damage to the plant is caused by the larvae living inside leaves and feeding on the sap and palisade parenchyma. Pupation takes place

*e-mail: danutakropczynska-linkiewicz@sggw.pl **e-mail: mfurmanowa@wp.pl

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inside the leaf. The leaf miner grows quickly and the female lays 20–40 eggs; there are three generations per year in Central Europe.

At high population densities of *C. ohridella*, leaves are completely covered with mines after feeding by the first generation, the population density of which increases so rapidly that trees are already infested with up to several hundred mines per leaf. As a result, early loss of foliage (long before autumn) takes place, followed by a second budding and refoliating. This impairs the winter frost hardiness of the horse chestnut tree (Balder et al., 2004).

Control of the horse chestnut leaf miner in urban areas is not an easy task. Insecticide spraying is limited under urban conditions, and in a number of towns forbidden because of environmental and health risks. Repetitive tree injections by which holes are drilled into the trunk can severely injure trees.

Work on alternative control strategies for the horse chestnut leaf miner is proceeding in a number of European research centers. One method being studied is to disturb host recognition and affect the egg laying of *C. ohridella* by applying plant-derived insecticides (botanical insecticides).

Secondary metabolites such as phenolic compounds, alkaloids, glycosides and terpenoids are known to be repellents and deterrents, as well as development regulators of many arthropod pests (Dąbrowski, 1988; Smith; 1989; Waligóra and Krzymańska, 1994; Felton and Gatehouse, 1996; Waligóra, 1998). They can play an important role in plant-pest interactions, especially as compounds related to the defense capability of infested host plants (Smith, 1989; Tomczyk, 1989, 2001; Felton and Gatehouse 1996; Kiełkiewicz-Szaniawska, 2003).

Extracts made from plants rich in secondary metabolites can be used as alternative pesticides in insect and mite control (Villani and Gould, 1985). The best known among those in commercial use is an extract made from *Azadirachta indica* containing azadirachtin and a closely related complex of alkaloids (Ascher, 1993).

Glycosylated sterols or terpenoids, known as saponins, can also act as insect deterrents or juvenile hormones, and affect their growth and reproduction (Ishaaya, 1986). For example, root extracts from alfalfa, rich in different saponins, demonstrated high insecticidal activity for *Spodoptera littoralis* (Adel et al., 2000). These extracts also disturb the development of the hairy rose beetle *Tropinota squalida* (Hussein et al., 2005). Saponin was also found to be correlated with the resistance of *Barbarea vulgaris* to the diamondback moth *Plutella xylostella* (Agerbirk et al., 2003). Natural saponins can also be used as a bioactive agent against insects transmitting human diseases: *Aedes aegypti* and *Culex pipiens* (Wiesman and Chapagain, 2003).

Bioactive saponin occurring in extracts of *Polyscias filicifolia* Bailey biomass can reduce the infection caused by *Staphylococcus areus* (Furmanowa et al., 2002). *Polyscias filicifolia* from the *Araliaceae* family is a known medicinal plant of tropical and subtropical climates, included in the National Vietnamese Pharmacopoeia. The plants are used as a tonic, adaptogenic and antimicrobial (Furmanowa et al., 2002). The dietary supplement Vitagmal containing *P. filicifolia* extract is accessible on the Russian market as a tonic, antistress specific and immunomodulator. This species has been cultivated in culture in vitro. Callus and suspension culture extracts from a bioreactor were studied using phytochemical methods: thin layer chromatography (TLC) and gas chromatography (GC). Oleanolic acid was found in biomass from the bioreactor and in callus growing on solid medium (Bloch, 2000).

The aim of this study was to determine the effect of *Polyscias filicifolia* extract in suppressing a *Cameraria ohridella* population on chestnut trees.

MATERIALS AND METHODS

CULTURE CONDITIONS

Cell suspension culture was initiated from callus of *Polyscias filicifolia* (strain BTF–001–95). The suspension was cultivated either in flasks agitated in a shaker or else in a bioreactor in modified liquid MS medium (Murashige and Skoog, 1962) supplemented with myo-inositol (0.08 g/l), casein hydrolysate (0.5 g/l), sucrose (3%), and the phytohormones kinetin (1 mg/l) and naphthaleneacetic acid (NAA, 2.0 mg/l). The pH of this medium was 5.0–5.5 after autoclaving.

Cultivation was conducted in darkness at 26 ± 0.5°C. Subculturing in shake-flask batch cultures was done every 14 days by adding 1 part 2-week-old suspension to 5 parts fresh medium. The rate of agitation was 80–100 rpm. The medium and equipment were sterilized by standard methods.

BIOREACTOR TYPE AND CHARACTERISTICS

Bioreactors of different types and volumes were employed for large-scale cultivation of *P. filicifolia* cells:

1. V-shaped glass bioreactor [original design; Department of Cell Biology and Biotechnology, Institute of Plant Physiology, Russian Academy of Science (IPP RAS)]; total volume 20–21 l; working volume 15 l.
2. Stirrer-jar fermenter (Electrolux, Sweden) with marine-type impeller; total volume 75 l; working volume 50 l.

TABLE 1. Main growth parameters for *P. filicifolia* cultivation in different culturing systems

Fermentation system	M_{max} (g/l)	V (%)	μ ($d.^{-1}$)	T (d)	P_{Mmax}
Shake-flasks	16	90 – 97	0.20 – 0.22	2.5 – 3.5	2.0
630L bioreactor 1T	13.2	85 – 95	0.12 – 0.14	2.0 – 2.4	0.7

M_{max} – maximum level of dry biomass, (g/l); V – cell viability (%); μ – specific growth rate in exponential phase ($day.^{-1}$); T(d) – time of biomass doubling (day); P – highest dry biomass productivity (g/l d).

3. Bioreactor 1T (OKB FARMBIOMASH, Yoshkar-Ola) with modified ring (Ø 750 mm); total volume 630 l; working volume 550 l.

Sterile compressed air was used for aeration.

GROWTH PARAMETERS

During cell cultivation, fresh (m) and dry (M) biomass were measured, and cell viability (V) was assessed. For bioreactors all these parameters were checked every 1–2 days for each subcultivation cycle.

The fresh weight of cultures was determined after all the excess medium was removed under vacuum with a Bunker's funnel. Then the samples were immediately dried at 60°C to constant weight for dry biomass measurement.

Specific growth rate in the exponential phase (μ), biomass doubling time (T) and highest dry mass productivity (P) were calculated as follows:

$$\mu = [\Delta \ln X_t/X_t + \Delta t]/\Delta t, (d^{-1})$$
$$T = \ln 2/\mu$$
$$P = (M_t - M_o)/\Delta t, (g/l\ d)$$

where X_{max}, X_t and X_o are the maximum, current and initial values of the corresponding parameter for each cell subcultivation cycle, respectively; M is dry biomass (g/l), and Δt is the time interval of exponential growth phase in days (d).

Cell viability was estimated as the percentage of unstained cells after mixing one volume of cell suspension with one volume of 0.025% Evans blue, counted under a dissecting microscope.

The pH value of the culture medium was measured with pH indicator paper and a pH meter.

In 2002–2005, several multicycles of semicontinuous *P. filicifolia* cell culture in 630 l barbotage bioreactors were performed. For scaling up, 20 l and 75 l bioreactors were used.

In all variants of the experiment, suspension was poured off and fresh nutrient medium topped up to equal the concentration of biomass (10–16 g/l for different cultivation cycles) corresponding to the beginning of cell growth slowdown. To exclude the lag phase, the suspension was diluted so that the concentration of dry biomass at the beginning of each cycle was no less than 3.5–4.0 g/l. Lag phases were noted at 2.5 g/l and lower. The characteristics of cell culture growth for a typical multicycle are given in Table 1. Cell biomass accumulation reached maximum when the inoculum/medium ratio was 1:3 and inoculum density was more than 4.5 g/l. The specific cell growth rate reached 0.14 d^{-1}, and maximum productivity as dry biomass was 0.7 g/l medium. Cell suspension viability went from 83–85% at the beginning of each growing cycle to 90–95% at the end. The data were similar for other cycles of semi flow-through culture technique or *P. filicifolia* cell suspension.

Scaling-up from standard cultivation in flasks on shakers to industrial-scale bioreactors did not decrease the main growth characteristics of cell culture.

PLANT EXTRACT PREPARATION

The dry biomass obtained from the bioreactor was extracted with 70% ethanol for 7 days in darkness according to the method described by Kašauskas and Vieželiené (2004). Then the alcohol was evaporated, and a 0.1% water solution was prepared and used for experiments.

Field experiments

Three experiments were conducted in 2004 on horse chestnut trees growing in Ursynow Park (Warsaw Agricultural University). For each experiment, four experimental and four control trees with 160–200 cm trunk girth were selected.

Experiment 1. To test the reaction of first-generation adult moths to plant extract, two white sticky traps were hung close to each other around the tree trunks 2 m above the ground, one painted with plant extract and one with water (Control I). On another group of four trees, sticky traps painted only with water were fixed (Control II). The number of adults caught was recorded after 24 and 48 h. The experiment was done 9 times during spring and early summer.

Experiment 2. Eight white sticky traps (35 cm × 10 cm) covered with the tested plant extract were placed in the canopy of four horse chestnut trees 2.5 m above the ground. Another 8 sticky traps

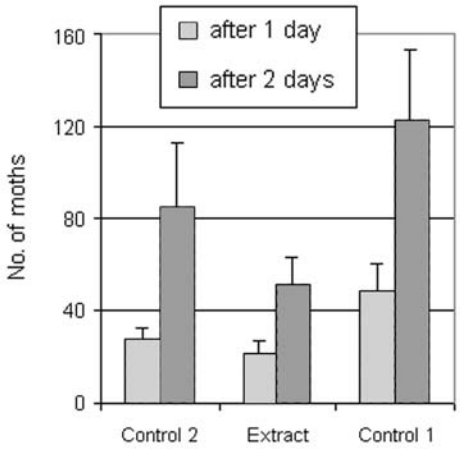

Fig. 1. Effect of *P. filicifolia* extract on *C. ohridella* moths when used on chestnut tree trunks. Control I – trap painted with water, hung on tree with trap covered with extract. Control II – trap painted with water on tree away from tree with extract-treated trap.

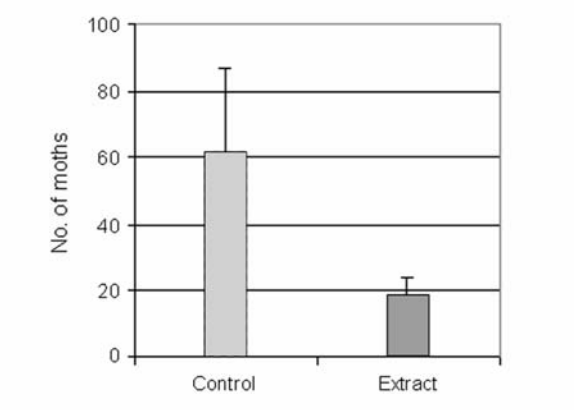

Fig. 2. Effect of extract from *P. filicifolia* on moths of *C. ohridella* when used on sticky traps hung in chestnut tree canopy.

painted only with water in the same trees served as the control. The trapping period was July 20–27. The cumulative number of catches during 7 days was recorded.

Experiment 3. At the end of May 2004, 24 leaves (six on each experimental tree) of similar size and age were selected and labelled. Two leaflets from each leaf were painted with *P. filicifolia* extract, and two situated opposite were painted with water only and served as the control. After 4 days the number of eggs laid by moths on treated and untreated leaflets was recorded.

Laboratory experiment

Twelve *A. hippocastanum* leaves were placed in vials with water and transferred to a growth chamber at 25°C under a 16 h photoperiod. Six of the leaves were left untreated, and the other six were painted on the upper side with the tested extract. Then the leaves were arranged alternately in three rows of four vials.

Fifty horse chestnut leaf miner adults of the second generation, collected at random from park trees, were introduced to the growth chamber. Mines made by horse chestnut leaf miner larvae were counted after four days.

RESULTS AND DISCUSSION

As shown in Figure 1, the extract obtained from *Polyscias* biomass used to cover the sticky traps on the trunks of chestnut trees had a repellent effect on *Cameraria ohridella* moths. The number of moths caught on the first day on the traps treated with extract was less than half that caught on the traps of Control II (trunks with traps painted only with water), and about 30% lower than for traps without extract hung on the same tree (Control I). The number of moths caught on all trunks increased the next day but was still much less on traps treated with extract.

The overall number of moth catches was higher on traps with the tested extract. The difference between the number of moths caught on treated and untreated traps on the same tree trunk was less than the difference between treated and untreated traps on different trunks. This indicates that the *Polyscias* extract can repel *C. ohridella* moths not only directly but also nearby.

Figure 2 makes it clear that in tree canopy the *P. filicifolia* extract had an even stronger repellent effect on *C. ohridella* moths than on the tree trunks.

Three times more *C. ohridella* moths were caught on traps treated with water than on those treated with extract. The data indicate that the behavior of the moths changed in the trees treated with extract. Even among the many leaves in the tree canopy they recognized the traps with extracts and avoided them.

The repellent effect of many plant secondary compounds is well documented and is known to be very important in the plant resistance mechanism of antixenosis (Felton and Gatehouse, 1996; Smith, 1989). Extracts of different plants rich in allelochemicals can be used against herbivorous insects and mites. *Azadirachta indica* extract is commercially used as an insecticide (Ascher, 1993).

The main secondary metabolites in *Polyscias filicifolia* extract are saponins. Oleanolic acid was

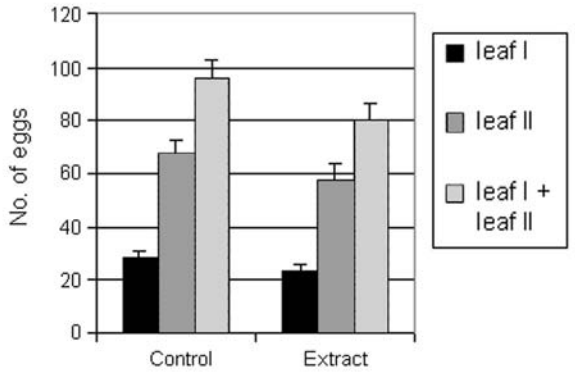

Fig. 3. Number of eggs laid by *C. ohridella* females on leaflets treated or not treated with *P. filicifolia* extract.

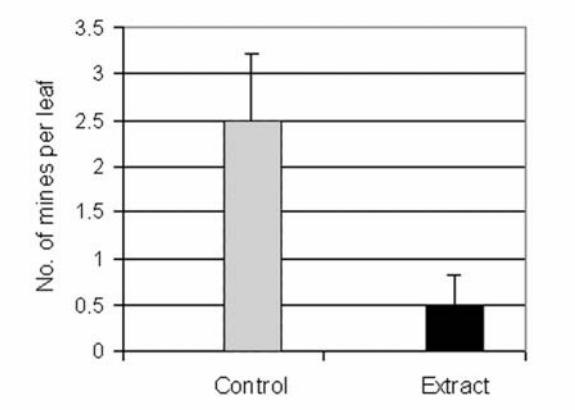

Fig. 4. Number of mines made by *C. ohridella* on leaves treated or not treated with *P. filicifolia* extract in laboratory experiment.

found, as aglicon, in many of them (Bloch, 2002). Saponins are known as insect repellents and deterrents (Adel et al., 2000; Agerbirk et al., 2003). The repellent effect of *Polyscias* extract on *C. ohridella* moths may be connected with this bioactive substance.

P. filicifolia extract also reduced oviposition on leaves treated with this extract (Fig. 3). On the leaflets treated with extract, *C. orhidella* females laid about 20% fewer eggs than on the untreated leaflets on the opposite side of the petiole. In that experiment the effect of *P. filicifolia* extract on oviposition was not as evident as the repellent effect on these moths. One possible reason for this is that the extract may have reduced the number of eggs laid on both the treated and untreated leaflets of the same leaf.

In the laboratory experiment, all chestnut leaves were treated either with extract or with water. The moths released in the growth chamber could make a choice between leaves treated with extract or with water. The number of small mines on the leaves was recorded for every leaf a few days later. The data are presented in Figure 4. In this experiment there was a large difference between the number of mines on the two groups of leaves. Single mines were observed on the leaves treated with extract (average less than 1), and 5 times more on control leaves.

In this case, as in the field experiments, the main reason for reduced symptoms of *C. ohridella* feeding on leaves treated with extract seems to be the repellent effect of extract on the moths (in this case only females). It is also possible that the mortality rate of hatched larvae was high on extract-treated leaves, so that leaf damage was not visible. Deterrent and toxic effects of allelochemicals (including saponins) are described in the literature (Felton and Gatehouse, 1996; Adel et al., 2000; Agerbirk et al., 2003).

The presented data indicate that *Polyscias filicifolia* extract can be used to repel moths congregating on chestnut trees, especially in springtime when the overwintering generation emerges from pupae. It disturbs the mating process and can reduce the number of eggs laid.

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