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Detrimental effects of water extracts from surface and interior of *Taxus baccata* leaves on the two-spotted spider mite (*Tetranychus urticae* Koch)

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Surface deposits on *Taxus baccata* needles removed by dipping in water of 96, 60 or 40 °C for 5 s caused changes in life history components of mites. Paclitaxel was among other peaks present in the removed fractions in concentrations between 0.017 and 0.170 μg/g of fresh weight (f.w.) increasing with temperature. Long extraction for 60 min at only 40 °C did not increase removable paclitaxel, but at 60 °C extraction rate was the highest (1.326 μg/g) suggesting that leakage from an interior of needles occurred. Mortality, developmental time, total fecundity, oviposition period and life history parameters of *Tetranychus urticae* Koch. were detrimentally affected.

1. Introduction

Taxus baccata L. (Sp. Pl. 1040, 1753) is the only species of the genus *Taxus* mentioned in Flora Europea [1]. It is a shrub or tree up to 20 m high with 10–30 mm leaves, dark, glossy green above, with two pale, green stomatiferous bands beneath. Seed 6–7 mm, $2n = 24$. The species has been cultivated for centuries with many cultures differing in habitat, leaf characters and colour. In Poland, besides *T. baccata,* other species and varieties cultivated in botanical gardens are *T. cuspidata*, *T. media* var. *Hicksii* and *T. media* var. *Hatfieldii*.

Taxus baccata is known as a source of paclitaxel, a diterpenoid having anticancer properties [2], which stabilizes microtubules and blocks cell replication in the late G-2 of mitosis [3]. The drug Taxol® (containing paclitaxel) was approved in 1992 by the US Food and Drug Administration for the treatment of ovarian as well as breast, lung and head cancers. Paclitaxel was isolated by Wani et al. [4] from the pacific yew (*Taxus brevifolia* Nutt.). It has been identified in different *Taxus* species and varieties (*T. baccata*, *T. cuspidata*, *T. media*) [5]. An interesting result was to find paclitaxel on the leave surfaces of the three *Taxus* species mentioned above [5]. Some other taxoids as well as other compounds could be detected on HPLC chromatograms [6, 7]. After dipping the needles in almost boiling water for 1 s we found that a concentration of paclitaxel on the surface in the range of 0.085–0.58%. After 5 s dipping at 95 °C a concentration of 0.170 μg/g was found [8] on *T. baccata* needles, thus substantial amounts which would be able to have antimitotic activity and cell divisions were retarded in the promeristem of root tips of *Allium cepa* L. [9]. Interior compounds could also be active but only when accessible after leakage or other kind of access to the interior of the cells, as for instance due to some insects chewing leaves [10]. *Taxus* is resistant to decay as well as to attacks of parasites and pests, so other surface or interior compounds, or both together, could be responsible for these phenomena. We have been interested in paclitaxel ecological activity. Previously, it appeared that a surface mixture including paclitaxel had an adverse effect on biological parameters of mites [11]. Here now we want to compare the reaction of surface mixtures removed by 5-s-dipping in almost boiling water, removing wax and compounds embedded in it, with longer submersion (60 min) in water of different temperature, which could cause leakage from vacuoles of epidermal cells, as well as from cells located deeper.

2. Investigations, results and discussion

Paclitaxel was identified among other peaks on HPLC chromatograms of *Taxus baccata* extracts. Its concentration was the lowest at the lowest temperature. The removal from the leaf surface by dipping for only 5 s increased with temperature, up to 10 fold when the temperature was increased from 40 to 96 °C. The maximum of surface paclitaxel removed by dipping in water was 0.170 μg/g of fresh weight.

Immersion for 60 min caused the extraction of concentrations ranging between 0.017–1.326 μg/g, which is an increase over 70 fold, when 60 °C water was applied. It was the lowest at 40 °C – like after 5-s-dipping, which suggests that in both cases the maximum of paclitaxel was removed. A temperature of 60 °C for a longer time allowed to obtain more paclitaxel, by at least a partial leakage from the interior.

All extracts caused distinct changes in life history components of the mites. Average developmental time was increased by 18–24% (Table 1). Mortality increased by at

Table 1: Paclitaxel concentration, as well as development, mortality and fecundity of *Tetranychus urticea* Koch. feeding on bean leaves treated with extracts from the needle surface of *Taxus baccata* isolated under different conditions

Taxus baccata	Temp. (°C)	Paclitaxel concentration µg/g (f.w.)	Mortality (%)	Mean developmental time (days)	Mean total fecundity eggs/female	Oviposition period (days)
5 s	96	0.170	19.2	13.36 ± 0.16	27.71	8.9
	60	0.136	25.8	12.92 ± 0.19	46.24	10.9
	40	0.017	13.6	12.09	50.41	11.2
60 min	60	1.326	18.0	13.49	39.88	9.9
	40	0.017	10.0	12.95	40.58	9.1
Control			14.0	10.90 ± 0.27	112.00 ± 18.76	17.5

least 20% except for both extracts obtained at 40 °C. Fecundity of females was drastically reduced by at least 75%. Oviposition period was shortened from 17.5 days (control) to 8.9–11 days. The obtained r_m value for mites feeding on plants treated with tested extracts ranged from 0.15 to 0.18, whereas for those feeding on control plants it was 0.27. According to Sabelis [12] the intrinsic rate of increase is a very good indicator of spider mite performance on different host plants. Its value reported for *T. urticae* ranged from 0.21–0.28 on good host plants and 0.05–0.12 on poor hosts [13]. The low r_m values of the mite population developing on extract treated leaves was related to the very low net reproductive rate (Ro) and a longer time of development. The mean generation time (T) for *T. urticae* on treated leaves was similar, except for the case of short extraction at 40 °C. In this case it was comparable to that obtained for the population developing on untreated leaves. The finite rate of increase (λ) shows the number of mites by which the population increases per specimen per day. The number of mites feeding on untreated leaves increased 1.31 times per day while on treated leaves 1.17–1.2 times (Table 2).

Even though paclitaxel is difficult to dissolve in water, all of our water extracts contained the drug among other compounds only partly identified so far. Paclitaxel was present in substantial concentrations between 0.017–1.326 µg/g of fresh weight thus comparable to values obtained by other researchers working on *Taxus* species [14]. Only the paclitaxel concentration obtained *in vitro* using methyl jasmonate was much higher. For these studies the HPLC method was used [15]. Results are presented in Table 3.

The increased concentrations of paclitaxel obtained by only brief dipping but increased temperature to almost boiling water can be used for compounds which are difficult to dissolve in water. Great amounts of furanocoumarins, up to ca 2 mg/g of fresh weight were removed [16] as compounds embedded in wax white melting the epicuticular layer. We found that a longer extraction time at 96 °C caused a leakage from cells located inside the leaves as observed after dipping *Ruta graveolens* for 10 s in almost boiling water [16, 17].

The conditions 60 min and 40 °C simulate warm rain forest conditions and 60 °C caused the removal of compounds from the interior of tissues – 1.326 µg/g, thus 8 times more than removed by dipping for 5 s at 96 °C. We could suspect as well that a longer period at 96 °C might have destroyed paclitaxel in our preliminary experiments. Thus it was not used in this trial.

Life history parameters of *Tetranychus urticae* Koch. feeding on bean leaves treated with extracts from *Taxus baccata* were detrimentally affected. Developmental time prolonged, mortality increased and a drastic decline in the number of eggs per female points that these compounds play a defensive role for *Taxus baccata*. Although oviposition values were lowered suggesting that compounds were not deterrents and could even be attractants, and did not prevent mites from approaching needles, harm was done

Table 3: Paclitaxel in twigs and callus of *T. media* var. *Hatfieldii* and transformed roots of *T. media* var. *Hicksii* by HPLC method

Plants growing in botanical garden	Paclitaxel concentration (µg/g dry weight)
Twigs of *T. media* var. *Hatfieldii*	12.66
Needles of *T. media* var. *Hatfieldii*	19.29

Material obtained *in vitro*	Without methyl jasmonate	After methyl jasmonate (100 µM)
Callus from twigs of *T. media* var. *Hatfieldii*	3.69	9.30
Callus from needles of *T. media* var. *Hatfieldii*	0	27.86
Transformed roots of *T. media* var. *Hicksii*	69.4	210.46

Table 2: Life history parameters of *Tetranychus urticea* Koch. feeding on bean leaves treated with extracts from needle surface of *Taxus baccata* isolated under different conditions

Taxus baccata	Temp. (°C)	Paclitaxel concentration µg/g (f.w.)	r_m – Intrinsic rate of increase	Ro – Net reproductive rate	T – Mean generation time (days)	λ – Finite rate of increase
5 sec	96	0.170	0.15	13.38	16.97	1.17
	60	0.136	0.17	21.83	21.83	1.18
	40	0.017	0.18	18.76	18.76	1.19
60 min	60	1.326	0.17	24.36	18.18	1.19
	40	0.017	0.15	18.52	18.84	1.17
Control			0.22	70.24	18.93	1.25

to mite population with the surface extract. Natural products on the surface of *Taxus* were found here to be an effective defense mechanism. Surface mixtures as a defense for plants could be useful for humans when used pharmaceutically, and different methods allowed us to remove different amounts of paclitaxel from the surface among other compounds. More analyses of other biologically useful compounds besides paclitaxel are needed. A possible synergistic reaction of those compounds and paclitaxel should be investigated.

3. Experimental

In this experiment a modified method was used which was first employed by Zobel and Brown [18] to extract the extruded furanocoumarins together with melted epicuticular waxes from *Ruta graveolens* leaf surface using almost boiling water [16, 19]. In the present experiment fresh needles (50 g) separated from two-year-old twigs of *T. baccata*, growing in the Warsaw Botanical Garden, were immersed in almost boiling water, or 60 °C, or 40 °C for 5 s to remove surface compounds only. Additionally, longer time (60 min) was used and temperatures of 40 or 60 °C were applied either to simulate warm-rain conditions (40 °C) or forming a possibile leakage because membranes could be damaged by protein modification at 60 °C. Taxoids removed together with melted epicuticular waxes were isolated from the water extract by dichloromethane (DCM) separation. The DCM fraction was evaporated to dryness and dissolved in 50% ethanol with 0.1% glacial acetic acid. Bean leaves as 2cm-diamenter-discs were painted with these extracts and the control with the solvent only. Mites were put on these discs 1 h after when leaf surfaces were dried.
Experiments were set according to the procedure described by Furmanowa et al. [11]. Results were compared using the non-parametric Kruskal-Wallis test.

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